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Modulation of T cell activation and Human Immunodeficiency Virus (HIV) infection by CD4: identification of functional domains and mechanisms involved in CD4 function

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1. Preface

The CD4 molecule is expressed on the surface of T cells that recognize antigen presented by MHC class II molecules. CD4 is associated with the protein tyrosine kinase $p56^{lck}$. lck expression is required for the initiation of T cell activation events such as tyrosine phosphorylation and increases in intracellular calcium. To study the function of the CD4/lck association in T cell activation, we generated a mutant of murine CD4 which is not associated with lck. This mutant and wild type CD4 were transfected into a murine CD4 negative T cell clone which recognizes the antigen ovalbumin presented by $I\text{-}A^b$ MHC class II molecules. The effect of this mutation on the function of CD4 was studied both at the level of generation of second messengers, and induction of proliferation following stimulation by anti-CD3 Ab, anti-TcR Ab and antigen.

SHORT TITLE

Modulation of T cell activation and Human Immunodeficiency Virus infection by CD4

ABSTRACT

The CD4 molecule is expressed on a subset of T lymphocytes which recognize their cognate antigen in the context of MHC class II molecules. It is widely accepted that the interaction between CD4 and MHC class II molecules enhances T cell response to specific antigen. CD4 is non-covalently associated with the src-related tyrosine kinase $p56^{lck}$. Using the T cell clone 2.10, we have shown that CD4 can sequester lck and inhibit anti-TcR induced proliferation if not co-aggregated with the TcR. Our results suggest that MHC class II molecules through their simultaneous interaction with the TcR and CD4 potentiate T cell activation by bringing the CD4/lck complex to the proximity of the TcR. This cellular system was also used to demonstrate that the extracellular domain of CD4 can also regulate the initiation of T cell activation independently of its interaction with MHC class II molecules. Indeed, cells expressing chimeric molecules composed of the epidermal growth factor receptor (EGFR) extracellular domain and the CD4 cytoplasmic tail were still responding to anti-TcR stimulation in the absence of co-aggregation. The role of the extracellular domain of CD4 was further demonstrated in experiments in which the HIV-1 envelope glycoprotein gp120 was used to inhibit antigenic stimulation of CD4-independent T cell responses. This inhibition was occurring whether CD4 is associated with lck or not, suggesting that gp120 is modulating a CD4 function other than association with lck and which is related to its extracellular portion. In addition to its effect on T cell activation, interaction between gp120 and CD4 modulates HIV replication at a post-transcriptional level. The CD4/lck association is required for this effect as the virus replicates much more efficiently in cells bearing a CD4 which is not associated with lck. Activation of lck through the CD4/gp120 interaction may thus be responsible for the induction of latency. Nef, another HIV protein playing a critical role in the positive regulation of HIV replication, induces internalization and targeting of CD4 to degradation in the lysosomes. We have examined the structural requirements in CD4 for this effect of Nef. Our mutagenesis results show that in addition to a di-leucine motif, an alpha helical stretch of amino acids in the cytoplasmic tail of CD4 is also involved in this down regulation. Interestingly, these residues are also implicated in the interaction of CD4 with lck and in the down regulation of CD4 by PMA. However, we have also identified mutants which can dissociate the PMA-induced and the Nef-induced internalization and degradation of CD4, confirming that these two phenomena occur via different pathways. In addition, we have shown that Nef also down regulates expression of CD4 by a second mechanism which occurs during the early steps of CD4 biosynthesis. This mechanism specifically targets the extracellular domain of CD4. Overall, the work described in this

thesis confirms the similarities in the regulation of T cell activation and HIV replication by CD4 and suggests that HIV has evolved to respond and modulate CD4 signals for its own purpose.

RÉSUMÉ

La molécule CD4 est exprimée à la surface des cellules T qui reconnaissent l'antigène présenté par les molécules de classe II du CMH. L'intéraction entre CD4 et une région conservée des molécules de classe II potentialise la réponse de ces cellules. Plusieurs modèles expérimentaux ont permis de démontrer que le rôle de CD4 est tributaire de l'association de CD4 avec la tyrosine kinase $p56^{lck}$. Nos résultat démontrent que l'effet de CD4 est aussi dépendent de sa fonction de co-recepteur, soit son aggrégation avec le TcR. La stimulation d'un clone T qui est indépendent de CD4 pour sa reconnaissance de l'antigène est inefficace si le TcR et CD4 ne sont pas co-aggrégés. Ces résultats suggèrent qu'une des fonctions de CD4 serait de prévenir une association fortuite entre lck et le TcR. La tyrosine kinase s'associerait au TcR suite à la reconnaissance simultanée de la même molécule de classe II par le TcR et CD4. Cette fonction requière non seulement le domaine intracellulaire de CD4 mais aussi sa portion extracellulaire. Ainsi, des cellules dérivées de ce clone exprimant une chimère constituée du domaine extracellulaire du récepteur EGF et de la portion transmembranaire et cytoplasmique de CD4 peuvent toujours répondre à une stimulation induite par le TcR et ce même en l'absence des molécules classe II du CMH. Cette fonction du domaine extracellulaire est de plus confirmée par des expériences d'inhibition de la réponse de ce clone par la protéine de l'enveloppe du VIH (gp120). Ainsi, des cellules exprimant une forme mutée de CD4 qui n'est pas associée à lck, ne répondent plus à l'antigène lorsque mises en présence de gp120. Ces expériences suggèrent que le domaine extracellulaire de CD4 pourrait de par son intéraction avec d'autres protéines exprimées à la surface de la cellule T moduler l'activation de la cellule T. La molécule CD4 peut aussi moduler la réplication du VIH de part son intéraction avec la protéine tyrosine kinase lck. Ainsi, le virus réplique plus efficacement dans des cellules exprimant des formes de CD4 qui ne sont pas associées à lck. Il est donc tout à fait possible que l'intéraction entre gp120 et son récepteur aboutisse à l'induction de la latence virale. Une autre protéine du VIH, Nef, qui joue un rôle primordial dans le contrôle de la réplication du virus, induit une diminution de l'expression de surface du CD4. Nous avons confirmé qu'un motif di-leucine est impliqué dans l'internalisation et le ciblage de CD4 vers les lysosomes. Par ailleurs, nous avons identifié une autre séquence de la portion intracellulaire de CD4 qui serait aussi impliquée dans l'effet de Nef sur l'expression de CD4. Cette séquence qui fait partie d'une hélice alpha est aussi impliquée dans l'association de CD4 avec lck. De plus, des formes mutées de CD4 nous ont permis de dissocier les mécanismes par lesquels Nef et la PMA induisent la dégradation du CD4. Finalement, nous avons démontré que Nef diminue l'expression du

CD4 par un second méchanisme. Cette inhibition a lieu au niveau de la synthèse du CD4 et implique le domaine extracellulaire du CD4. En conclusion, nous avons défini et charactérisé les fonctions régulatrices du CD4 et de l'association CD4/lck impliquées dans l'initiation de l'activation de la cellule T et la réplication du VIH. Nos résultats démontrent que le VIH a évolué afin de pouvoir être susceptible aux signaux générés à travers CD4 et de les moduler pour ses propres fins.

PREFACE

The Guidelines Concerning thesis preparation of the Faculty of Graduate Studies and Research of McGill University reads as follows:

"Candidates have the option, **subject to approval of their Department**, of including, as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis.

- If this option is chosen, **connecting texts, providing logical bridges between the different papers, are mandatory.**
- The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in a literary from that is more than a mere collection of manuscripts published or to be published. **The thesis must include, as separate chapters or sections:** (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature to the subject of the thesis, when this review is appropriate, and (5) a final overall conclusion and/or summary.
- Additional material (procedural and design data, as well as descriptions of equipment used must be provided where appropriate and in sufficient detail (e.g. in appendices) to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.
- In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent:** supervisors must attest to the accuracy of such claims at the Ph.D. Oral Defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate 's interest to make perfectly clear the responsibilities of the different authors of co-authored papers."

My thesis will be presented according to the option mentioned and consists of 8 chapters. Chapter 1 is a general overview of the background literature. Chapter 2 is a paper that was published in Nature and chapter 5 was published in EMBO Journal. Chapters 6-7 are submitted for publication. Chapter 3-4 are manuscripts in preparation. Chapter 8 consists of a gereral discussion.

The contribution of each co-authors in the manuscripts presented are as follows:

1. Haughn L., Gratton S., Caron L., Sékaly R.-P., Veillette A., Julius M. Association of Tyrosine Kinase $p56^{lck}$ with CD4 Inhibits the Induction of Growth through the αβ T cell Receptor. Nature 358:328-331 (1992).

The T cell clones used in this study were derived in the laboratory of Dr. Julius. I actively participated in the elaboration of the experiments. I performed all the mutagenesis and some of the transfections. I calibrated calcium measurements in these clones and performed with L. Haughn the experiments on calcium reported in the paper. L. Haughn performed the functional and biochemical results. L. Caron and A. Veillette performed preliminary experiments which were not shown in the paper. The manuscript was written as a collaborative effort between all the co-authors.

2. Gratton S., Haughn L., Sékaly R.-P, Julius M.. The Extracellular Domain of CD4 Regulates the Initiation of T Cell Activation. (In preparation)

The experiments were elaborated by R.-P. Sékaly and myself with suggestions from the other co-authors. I performed all the experiments reported in this paper: mutagenesis, transfections, functional assays and biochemical experiments. I wrote up the manuscript which was revised by R.-P. Sékaly. The original clone used for the transfection was donated by L. Haughn and M. Julius.

3. Gratton S., Haughn L., Sékaly R.-P., Julius M. lck Independent Inhibition of Antigenic Stimulation by the HIV gp120. (In preparation)

All of the experiments were elaborated by R.-P. Sekaly and myself, with suggestions from the other co-authors. I performed all the experiments reported in this paper: mutagenesis, transfections, functional assays and biochemical experiments. I wrote up the manuscript which was revised by R.-P. Sékaly. The original clone used for the transfection was donated by L. Haughn and M. Julius.

4. Tremblay M., Meloche S., Gratton S., Wainberg M.A., Sékaly R.-P. Association of $p56^{lck}$ to the Cytoplasmic Domain of CD4 Modulates HIV-1 Expression. EMBO J. 13:774-783 (1994).

I participated directly to the elaboration of the experiments. I generated the mutants used and some of the stable transfectants used in this paper. I performed the analysis of lck activity in the transfectants. M. Tremblay performed the HIV infections and related experiments as a post-doctoral fellow in the lab. After he left, I took over the project on my own and performed the experiments which were required for the acceptation of the

paper after revision. I had a major contribution in the writing of the manuscript. S. Meloche, M.A. Wainberg and R.-P. Sékaly participated in the elaboration of the experiments and in the writing of the paper.

5. Chandrasekhar S.*, Gratton S.*, Popov S., Broder C., Sékaly R.-P., Venkatesan S. HIV-1 Nef Down Regulates the CD4 Receptor by a Bi-Modal Mechanism. Submitted to Journal of Virology. * the authors contributed equally to the work.
This work results from a collaborative effort between our lab and the lab of Dr. S. Venkatesan at the NIH. I personally went to the NIH to perform most of the experiments. The mutants and cell lines used were generated by myself. Dr. Popov calibrated the conditions for vaccinia infection and Dr. Chandrasekhar performed some of the pulse labeling studies. The writing of the paper was a collaborative effort mainly between R.P. Sékaly, S. Venkatesan and myself.

6. Gratton S., Yao X.-J., Venkatesan S., Cohen E., Sékaly R.-P. Molecular Analysis of the Cytoplasmic Domain of CD4: Overlapping but Non-competitive Requirement for lck Association and Down regulation by Nef. Submitted to Journal of Virology.
This work results from a collaborative effort between the lab of Dr. Cohen and our lab. I elaborated and performed all the experiments. Dr. Yao generated some of the mutants used in this study and helped with the HIV infections. Dr. Venkatesan helped in the analysis of the results. The manuscript was written up by myself and Dr. Sékaly.

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I am very grateful for the support, numerous interesting discussions, and excellent advice received from my supervisor, Dr. Rafick-Pierre Sékaly. He is a source of inspiration and a role model for any young investigator. His ambition and his passion for science are easily transmitted to others.

I wish to specifically acknowledge Jacques Thibodeau for his continued support through the ups and downs of the last five years. I could not have achieved this without him.

I thank all the members and ex-members of the laboratory of Immunology at the IRCM, for helpful discussions, support and friendship. I will remember for a long time the interesting meetings of the CD4 group. I especially am grateful to Jacques Bernier.

I wish to thank Nicole Guay for her expert secretarial help, Claude Cantin for sorting all my clones (even the negative ones!) and for performing the calcium and cell cycle analysis. My deepest gratitude goes to Jean-Pierre Fortin for everything, including killing my mice, and for being a friend too.

I extend my gratitude to Dr. H. Goldsmith for his support and encouragements throughout my Ph.D.

I acknowledge all our collaborators, especially Dr. E. Cohen and Dr. X.J. Yao from Université de Montréal, Dr. S. Popov, Dr. Chandrasekhar and Dr. S. Venkatesan from the NIH, and Dr. Michel Tremblay in Québec.

I am indebted to the MRC for financial support during the last five years.

To my husband Jacques,

To my parents,

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List of abbreviations

Ab	Antibody
Ag	Antigen
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
ARAM	Antigen recognition activation motif
CDR	complementarity determining region
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
FITC	Fluorescin
FCS	Fetal calf serum
GAM	Goat anti-mouse
gp	glycoprotein
HLA	Human leukocyte antigen
HIV	Human immunodeficiency virus
Ig	Immunoglobulin
IL-2	Interleukin 2
ITAM	Tyrosine-based activation motif
Kb	kilobase
kDa	kilodalton
LTR	Long terminal repeat
mAb	Monoclonal antibody
mRNA	messenger RNA
mwt	Molecular weight
MHC	Major histocompatibility complex
ORF	Open reading frame
PBL	peripheral blood lymphocytes
PBS	Phosphate buffered saline
PKC	Protein kinase C
PLC	phospholipase C
PE	Phycoerythrin
RNA	ribonucleic acid
SIV	Simian immunodeficiency virus
SCID	Severe combined immunodeficiency disease

SDS	Sodium dodecyl sulfate
TcR	T cell receptor
V	Variable region of the T cell receptor

CHAPTER 1

INTRODUCTION

1. THE IMMUNE SYSTEM: A BRIEF OVERVIEW

The mammalian immune response can be divided into the non-specific inflammatory response and the specific response. The inflammatory process is the first line of defense of the organism and involves various cell types such as the monocytes/macrophages, the neutrophils, the eosinophils, the basophils and related cell types. Another important player in this process is the complement cascade which can also lead to direct lysis of targets.

The mediators of the specific arm of immunity are the lymphocytes. B lymphocytes are responsible for the humoral response , i.e. the immunoglobulin (Ig) production. Cytotoxicity can be mediated by NK cells as in the lysis of tumor cells or by cytotoxic T lymphocytes bearing specific T cell receptors for a given antigen. Both the Ig production and the specific cytotoxic response are regulated by helper T cells whose mediators are cytokines such as the interleukins. Subsets of T lymphocytes can be differentiated on the basis of cell surface markers: helper T cells are generally CD4 positive whereas cytotoxic T cells express the CD8 molecule at their surface.

The most studied model is the mouse for convenience and the availability of inbred strains of mice. The human immune system however seems to work and to be highly related to the murine system as many human molecules can substitute for their murine counterparts in murine cells.

T cell activation initiates with the recognition of the peptide/MHC complex by the α/β chains of the T cell antigen receptor (TcR). This interaction results in a rapid increase in tyrosine phosphorylation of several cellular proteins, leading to activation of other second messengers such as serine/threonine kinases (MAP kinase, raf). Phospholipase C γ (PLCγ) is rapidly serine and tyrosine phosphorylated following TcR ligation leading to its activation and generation of phosphatidyl inositols (PI) (66,226). This results in an increase in intracellular calcium and activation of protein kinase C (PKC). Activation of the ras protooncogene was also demonstrated to occur and may implicate the recently identified signaling protein called vav (77,113). Ultimately, transcription of a vast number of activation genes is induced. These include cell surface markers such as CD69 and the IL-2 receptor alpha, cytokines such as IL-2 and a number of protooncogenes such as c-myc (238).

Resting T cells are in the G0 phase of the cell cycle. Recognition of antigen induces progression to the G1 phase of the cycle. This is accompanied by an increase in size of the activated cell and induction of transcription of the IL-2 gene. The secretion of IL-2 will act in an autocrine and/or a paracrine way to stimulate cell cycle progression in

the S phase. Activated T cells then divide and the continuing stimulation by IL-2 results in clonal expansion.

The outcome of antigenic stimulation depends on the presence of a co-stimulatory signal. Ligation of the TcR alone does not stimulate proliferation of the T cell but rather results in a state of non-responsiveness termed anergy (269). This state of anergy is characterized by a lack of proliferation of the stimulated T cells which seems to be due to a lack of IL-2 production. The generation of second messengers following T cell ligation such as tyrosine phosphorylation and phosphatidyl inositols (PI) generation, is impaired in anergic cells (97,163). The most potent co-stimulatory signal is delivered through the interaction between CD28 at the surface of T cells and a member of the B7 family (B7-1, B7-2) which are expressed on the surface of APCs (18,95,119,240). Several interactions between other cell surface molecules on the T cell and the antigen presenting cell (APC) such as ICAM/LFA-1 and CD2/LFA-3, can act as a partial costimulatory signal as they can deliver a co-signal for proliferation and cytokine production, but the resulting T cells are anergic (31,63).

The purpose of this thesis was to study the signaling function of the CD4 molecule. In the first chapters, we have studied the normal function of CD4 in regulating the initiation of T cell activation. We then went on to study the role of CD4 in a pathological situation, i.e. in HIV infection. Consequently, I will first review in the introduction the presentation of antigen to T cells, the development of T cells and the first events in T cell activation, more particularly the role of CD4 in these events. The second part of the introduction will focus on the human immunodeficiency virus (HIV), with an emphasis on HIV proteins which interact with CD4.

2. MHC RESTRICTION AND ANTIGEN PRESENTATION

T cells recognize antigen presented in the form of a peptide associated with Major Histocompatibility Complex (MHC) molecules (reviewed in 103). Generally, CD4 positive T cells recognize peptides presented by MHC class II molecules whereas CD8 positive T cells recognize antigen presented by MHC class I molecules. An individual cell expresses three MHC class II isotypes in the human (DR, DP, DQ) and 2 in the mouse (I-A and I-E), and up to 3 different classical class I molecules in humans (A,B,C) and 2 in the mouse (K,D). The MHC locus is polygenic and highly polymorphic. This polymorphism is mainly located in the peptide-binding groove and allows the host to respond to a wide variety of antigens. The response of T cells is MHC restricted in that the antigen specific

receptor on T cells recognizes specific residues both on the antigenic peptide and on the MHC molecule.

MHC class I molecules are heterodimers composed of a 45 KDa MHC-encoded alpha chain and a 12 KDa non-MHC encoded soluble protein, β-2 micro globulin. Peptides presented by class I molecules are derived from endogenously synthesized proteins, either cellular or from intracellular parasites such as viruses. Almost all cells in the body express MHC class I molecules although levels of expression vary and are typically higher on cells of the hematopoetic lineage. CD8 positive T cells, generally cytotoxic, can thus recognize infected cells which present foreign peptides associated with class I molecules at their surface and lyse them. The binding site of the peptide is located on the α chain as a pocket formed by two α helices underlined by a β sheet floor. Peptides associated with class I molecules are usually between 9 and 11 amino acids long. Association of the peptide with the α chain occurs in the Endoplasmic Reticulum (ER). Studies have demonstrated that the α chain is associated with calnexin or p88 in the ER which retains the class I until it associates with a proper peptide and β-2 micro globulin. The peptide stabilizes the heterodimer and allows its egress from the ER to the Golgi apparatus. The generation of peptides which are associated with class I molecules involves a large cytoplasmic complex called the proteasome. Two of the subunits of the proteasomes, LMP-2 and LMP-7, are encoded within the MHC locus and are also polymorphic. Peptides are then translocated from the cytoplasm to the ER by ATP dependent transporters encoded by the TAP-1 and TAP-2 genes. Expression of these transporters was found to be essential for association of peptides with class I molecules and subsequent expression of class I at the cell surface leading to presentation of antigen to T cells. The genes coding for these transporters are also polymorphic, thus allowing size and sequence variability of the transported peptides.

MHC class II molecules rather present peptides derived from exogenous proteins. Unlike the class I, MHC class II expression is restricted to professional antigen presenting cells such as B cells, dendritic cells, macrophages and monocytes. Interferon-γ (INF-γ) was demonstrated to induce class II expression on a wide selection of cell types. CD4 positive T cells are generally helper T cells and upon recognition of antigen, secrete cytokines such as IL-2, IL-4, INF-γ, etc..., which stimulate the immune response of cytotoxic T cells and drive B cell differentiation and proliferation. Class II molecules are heterodimers composed of a 33 KDa alpha and a 29 KDa beta chain both encoded within the MHC. Both of these chains participate in the peptide binding. The binding pocket of the class II is also defined by two α helices underlined by a β sheet floor, but can accommodate much larger peptides than MHC class I molecules. MHC class II molecules

associate in the ER with a chaperone , the invariant chain. In fact, trimers are formed in the ER consisting of three alpha, three beta and three invariant chains. The invariant chain facilitates the formation of heterodimers, targets the class II molecules to the endosomes where class II molecules associate with peptides and also prevents peptide loading of class II molecules before they reach the endosomes by occluding the peptide binding groove. These functions were clearly demonstrated in invariant chain deficient mice which had a markedly diminished cell surface expression of class II molecules. Once the complex has reached this compartment, the invariant chain is cleaved off the class II and peptide is loaded into the groove. The peptide class II association is very stable and long lived.

3. THE T CELL RECEPTOR COMPLEX

T cells recognize the peptide-MHC complex through their antigen specific receptor. Two types of T cell Receptor (TcR) can be expressed on the surface of T cells in a mutually exclusive manner: the α/β and the γ/δ TcR. The role, localization and maturation of these two types of T cells differ. Since the results presented in this thesis only concern α/β T cells, no further description of the γ/δ T cells will be given. The α/β TcR is a di-sulfide linked heterodimer composed of an α chain and a β chain both members of the Ig superfamily (figure 1). One T cell expresses generally one clonotypic receptor, although some exceptions have been reported recently (69,223). Several mechanisms are involved in generating the diversity of the TcRs so that the repertoire of T cells is as vast as possible and is able to recognize virtually any antigen (reviewed in 264). The chains of the TcR are generated by gene rearrangement and both contain a variable domain which is involved in antigen recognition, and a constant domain. The variable domain of the α chain results from the rearrangement of a Vα(variable) and a J (joining) region. The β chain consists of a Vβ region, a D (diversity) region , and a J (joining) region. In humans, more than one hundred Vα genes classified into twenty nine families, and about sixty five Vβ genes grouped in twenty four families, have been identified up to now. The genotypes and the rearrangement of the TcR genes thus contribute to increase the diversity of the TcR repertoire. Moreover, addition of nucleotides at the rearrangement sites by a terminal deoxy transferase (TdT) or their deletion further contribute to the diversity of the TcR repertoire. Taken together, this allows a possible $10^{8\text{-}9}$ different TcRs to be expressed, thus allowing the recognition of the same number of antigens. Rearrangement of the α and the β chains occurs in the thymus as T cells mature (see below) and requires the expression of two DNA recombinases, RAG-1 and RAG-2 (205,263,277).

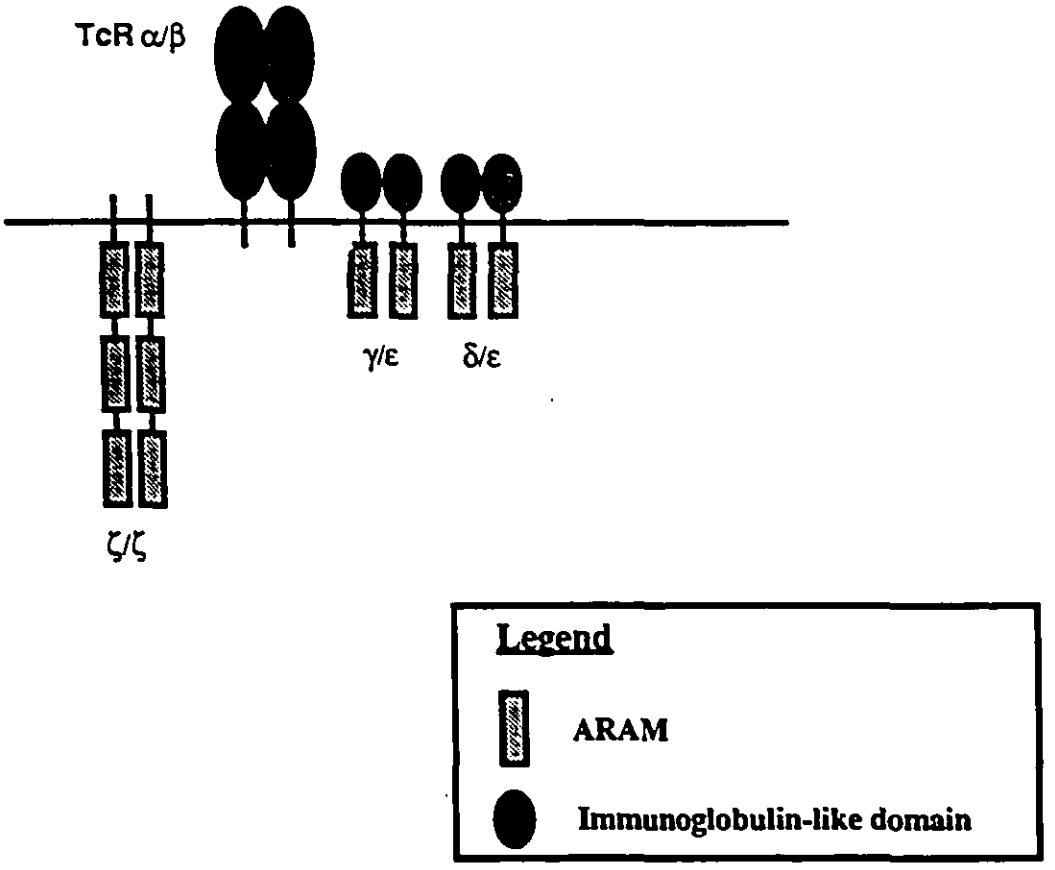

Figure 1. Organization of the T cell receptor/CD3 complex.

The T cell receptor (TcR) is associated non-covalently with the CD3 chains and a dimer of the zeta family. These non-polymorphic chains are responsible for the generation of signal transduction following antigen recognition by the TcR. The ARAM sequences contained in the cytoplasmic domain of these chains mediate the initiation of T cell activation.

As mentioned above, the recognition of the peptide/MHC complex is achieved by the combination of the Vα and the Vβ domains. There are three hypervariable regions on both chains, the CDR1, CDR2 and CDR3, which define this recognition process. Elegant studies have demonstrated that the CDR3, which is encoded by the D-J and V-J junctions , can directly interact with the presented peptide (65,84,139). In this model, the CDR1 and CDR2 recognize residues on the MHC molecule which point upward toward the TcR. Whether all the TcRs interact with the peptide/MHC complex in the same orientation and involving the same amino acids is still being characterized presently.

The cytoplasmic domains of the α and the β chains are short (5 amino acids) and lack any known enzymatic activities that could transmit a signal upon antigen recognition to activate the T cell. The signal transduction is rather mediated by a multisubunit complex which includes the CD3 proteins (γ,δ and ε) and a dimer of the zeta protein family, and which is associated non-covalently with the TcR (figure 1). The exact stochiometry of the TcR/CD3 complex is not known but the current model is that there are one α and one β chains, associated with a δ/ε dimer, a γ/ε dimer and either a ζ/ζ homodimer or a ζ/η heterodimer (234). The mechanisms by which signal transduction is initiated and leads to T cell activation will be discussed below.

The ε, δ, and γ chains possess an Ig-like extracellular domain, whereas the ζ and η chains only have 9 extracellular amino acids. All chains have a single transmembrane domain and a cytoplasmic domain ranging from 40 to 113 amino acids. The η chain results from alternative splicing from ζ mRNA, giving rise to a molecule which is truncated at the C-terminal portion. The pattern of expression and functional relevance of this alternate splicing is not known. In humans, studies have demonstrated that less than 0.25% of ζ RNA was spliced into η whereas in mice, up to 10% of ζ mRNA can be spliced into η (138). Studies using T cell hybridomas expressing varying amounts of homo- or hetero-dimers have given contradictory results on the role of these two splicing products in the generation of second messengers (22,192,193).

The CD3 and ζ chains also regulate the expression of the TcR (187,198). Indeed, the TcR α/β must be associated with the CD3 chains and ζ dimer in order to leave the ER. Unassembled or partly assembled complex are retained and degraded in the ER. The association of the TcR with the CD3 is mediated through basic residues in the transmembrane domains of the α and β chains interacting with acidic residues in the transmembrane of the CD3 chains.

4. THYMIC MATURATION

T cells undergo maturation in the thymus. The goal of this process is to select cells expressing a TcR able to recognize foreign peptides associated with self MHC (positive selection), and to eliminate autoreactive T cells which express a TcR which recognizes self peptides associated with self MHC and that can thus lead to autoimmunity (negative selection) (reviewed in 215 and 319). This maturation is accompanied by a tremendous amount of cell death; over 95% of the thymocytes are eliminated due to negative selection or to a lack of positive selection. Studies have implicated apoptosis as a mechanism for cell death.

A long list of cell surface markers has been described that define the steps of maturation of T cells in the thymus. Only the markers of interest to this thesis will be discussed. T cells that enter the thymus are $CD4^-CD8^-TcR^-$. The TcRβ locus is the first one rearranged and upon productive rearrangement, the β chain is expressed at the surface associated with a surrogate α chain, the gp33 (253). Evidence suggests that this step is a checkpoint to ensure that a productive β rearrangement has occurred and that development can continue. This checkpoint also appears to be linked with allelic exclusion, which allows only one α and one β chain per T cell to be expressed. However, the mechanism by which the gp33/β dimer signals is still obscure. Once the TcRα locus has successfully rearranged, the thymocytes start to express both CD4 and CD8. At this stage, the TcR levels are low. The transition from double negative ($CD4^-CD8^-$) to double positive ($CD4^+CD8^+$) is accompanied by a strong proliferation of the thymocytes. The double positive cells ultimately will mature into single positives, expressing either CD4 or CD8 and high levels of TcR.

Positive and negative selection have been found to occur at different stages of maturation depending on the system. Experiments using mutant mice have demonstrated that expression of MHC class I is required to get mature $CD8^+$ T cells (150,339) and that MHC class II expression is required to get mature $CD4^+$ T cells in the periphery (58,112). Conversely, the study of mutant mice lacking expression of either CD4 or CD8 suggested that CD4 expression is required to select MHC class II restricted cells (236), whereas CD8 expression is required to select MHC class I restricted T cells (96). Several models have been put forward to explain the mechanisms of positive and negative selection. The current favored hypothesis is that the avidity of the TcR for the antigen/MHC complex will dictate whether the thymocyte is positively selected or negatively selected.

5. T CELL ACTIVATION

5.1 Initiation of T cell activation

Stimulation of T cells by antigen can be mimicked by cross-linking of the TcR using anti-TcR or anti-CD3 antibodies, suggesting that aggregation of TcR by MHC molecules initiate activation. This is reminiscent of what is occurring in growth factor receptors where binding of the growth factor to its receptor induces its dimerization leading to activation of its tyrosine kinase activity and transmission of the mitogenic signal (307). However, this does not exclude the possibility that a conformational change in TcR upon antigenic stimulation also generates intracellular signalling. It was demonstrated that a series of anti-TcR Abs directed to different epitopes on T cells lead to differential activation signals (270). Furthermore, recent studies in which T cells were stimulated using peptide analogs also support a conformational constraint for signal generation through the TcR. These stimulations resulted in partial signaling at the level of tyrosine phosphorylation events, and in induction of cytokine production without proliferation, or upregulation of cell surface markers without proliferation or cytokine production (87,88,251,284,285). Moreover, the resulting T cells were anergic to restimulation. These observations thus suggest that subtle changes in TcR ligands can induce differential signaling and that activation of T cells does not merely require aggregation of TcRs.

The first detectable event following TcR ligation is an increase in intracellular tyrosine kinase activity (140). A number of proteins become phosphorylated upon ligation of the TcR, including the CD3 δ, γ and ε chains and the ζ chain (235). Two families of tyrosine kinases are involved in this process: the src-related tyrosine kinases (lck, fyn) and the Syk family (syk, ZAP). The importance of tyrosine kinases in the initiation of T cell activation was demonstrated by studies using tyrosine kinase inhibitors which completely abrogated the initiation of T cell activation (141,289). Moreover, deficiency in expression of either the ZAP or the lck tyrosine kinase (see below) leads to a state of non-responsiveness of the T cell. On the other hand, overexpression of activated forms of lck or fyn renders T cells hypersensitive to anti-TcR stimulation. Further evidence for the importance of tyrosine kinase activity is the requirement for CD45, which is a tyrosine phosphatase implicated in the activation of src-related tyrosine kinases such as lck and fyn. The mechanism by which tyrosine kinases are activated and lead to signal transduction will be discussed below.

5.2 The CD3 and ζ chains as signal transducers

Confirmation that the CD3 chains were responsible for signal transduction came from a series of studies which used chimeric molecules between the extracellular domains of cell surface molecules such as CD8, CD4, the IL-2 receptor α chain, and the cytoplasmic domains of ζ or ε (85,133,170,246,247,324). Cross-linking of the chimeras resulted in the induction of early activation signals such as tyrosine phosphorylation and increase in intracellular calcium, as well as later events such as IL-2 production and induction of CD69 expression. The activation resulting from the cross-link of the chimeras was identical to that observed following ligation of TcR. A 17 amino acids motif (D\EXXYXXL-X(6-8)-YXXL) was identified in the cytoplasmic domains of the CD3 chains and ζ to be responsible for signal transduction. The ζ chain possesses three copies of this motif and the γ, δ and ε chains each contain one copy of the motif. The signalling motif, termed ARAM (for antigen recognition activation motif) or ITAM (for immune receptor tyrosine-based activation motif), was found to be present in a number of signal transducing subunits associated with hematopoetic cell antigen receptors, such as the FcγRI, and the α and β chain associated with the membrane Ig. The genetic organization of the exons coding for these motifs suggest that they arose from a common ancestor sequence. Interestingly, functional ARAM sequences were found in the cytoplasmic domain of viral proteins from the EBV and BLV viruses, both of which are involved in B cell transformation (23).

Importantly, it was demonstrated that one copy of the ARAM is able to transduce the necessary signals required for T cell activation in cell lines (132). This was demonstrated by chimeras with CD3ε (1 ARAM) and chimeras with truncated forms of ζ which induced T cell activation upon cross-linking albeit to lower levels than that elicited by a chimera with full length ζ. Since one TcR can be associated with up to ten ARAMs (6 ζ, 2 ε, 1 δ, 1 γ) (figure 1), one interesting question is why the apparent redundancy? Several pieces of evidence seem to point to a mechanism of amplification of the response. It was reported that only a few hundred receptors are likely to be engaged in antigen recognition, so it would be logical that TcR possess a sensitive signal transduction system (310). Mutant mice lacking the expression of ζ were found to be severely impaired in thymic maturation but this could be accounted for by the lack of expression of cell surface of TcR (177,185,217). Interestingly, re-expression of a mutant ζ defective in transduction in these mice restored thymic maturation (279). These results indicated that the presence of ζ is required for proper cell surface expression of the TcR/CD3 complex but that the presence of multiple signalling motifs in the CD3 complex may only be responsible for amplification of the signal and not differential signalling. Moreover,

certain T cell hybridomas expressing a defective ζ could be stimulated with antigen or superantigen to produce IL-2 further suggesting a redundancy (94,123).

Other experiments lead to the concept that the TcR/CD3 complex was composed of two autonomous transduction modules, the γ/δ/ε and the ζ modules (324). Activation through the CD2 and Thy-1 pathways required expression of the ζ module, demonstrating that the modules could deliver non-overlapping signals. However, recent evidence suggest that this differential signalling is not observed in all T cell hybridomas (123). In addition, the pattern of tyrosine phosphorylation induced by ligation of the CD3ε and ζ chimeras are different although both are associated with the ZAP kinase. Moreover, the raf serine kinase which is activated upon TcR ligation, associates with the CD3 δ and γ only, and not with CD3 ε or the ζ chain (176).

Taken together, the CD3 organization seems to favor amplification of signals but there is specificity in the signal generated by individual chains which is not yet completely understood. These could be involved in thymic selection and differential induction of activation genes such as cytokines.

5.3 Tyrosine kinases in TcR signal transduction

The first biochemical event detected following ligation of the TcR is an increase in tyrosine kinase activity. Three tyrosine kinases have been associated with this event using both biochemical approaches and genetic approaches. Two are src-related cytoplasmic tyrosine kinases, p56lck and p59fyn. The other, ZAP 70, is a member of a recently discovered family, the Syk family of tyrosine kinases.

5.3.1 src-related tyrosine kinases

Members of the src family have several well-studied structural features (reviewed in 57) (figure 2). 1) These cytoplasmic tyrosine kinases are myristylated at a glycine residue at position 2 of their N-terminus. This allows their membrane association which is required for their biological activity. 2) A unique stretch of about 80 amino acids in the N-terminal portion of the kinase defines specific interaction of the kinase with other proteins. 3) Three domains are shared by all src-related tyrosine kinases, the SH2, the SH3 and the kinase domain. The SH2 domains (for src homologous domain 2) contain about 100 amino acids and interact specifically with phosphotyrosine residues. Proteins

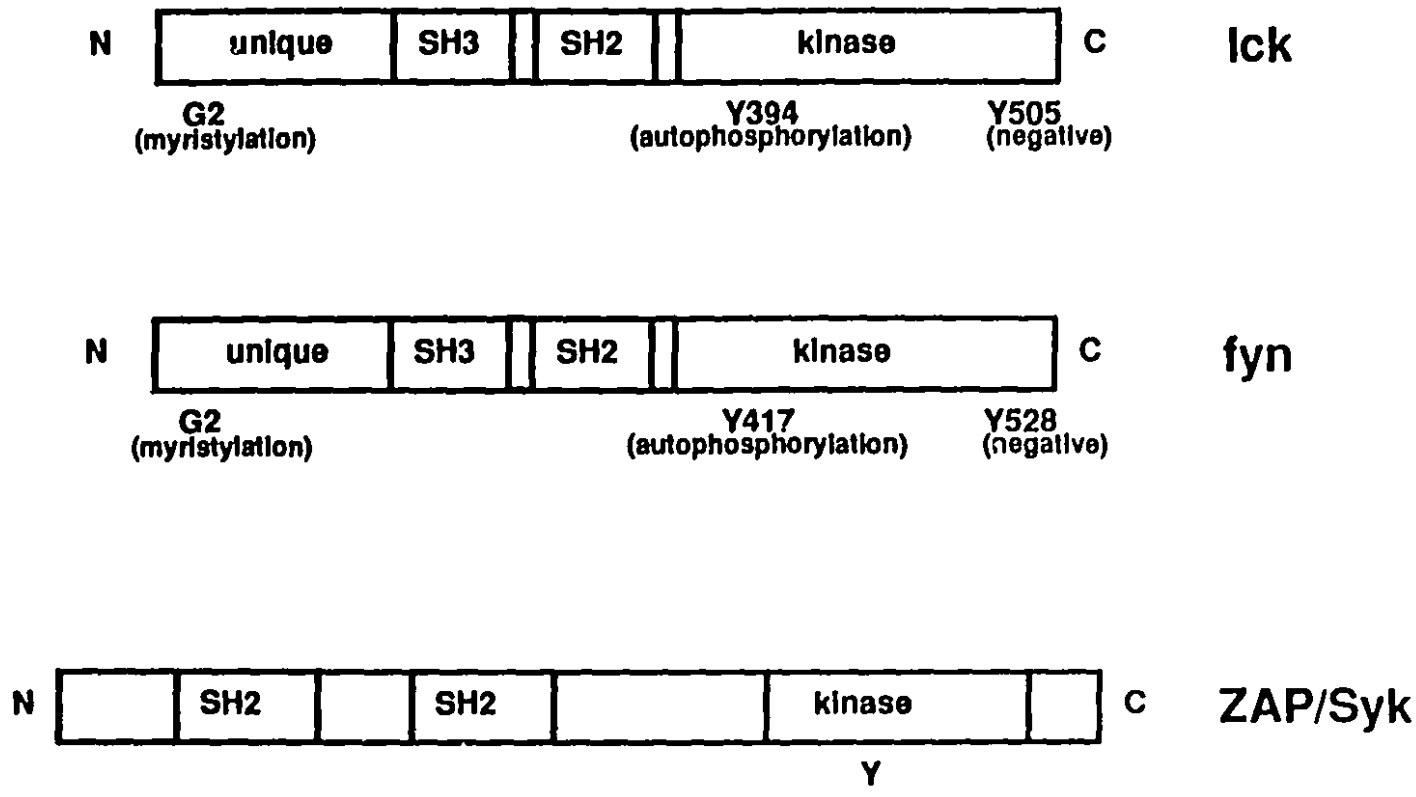

Figure 2

Structure and functional domains of the tyrosine kinases implicated in T cell activation

involved in signal transduction use their SH2 domains to associate with each other and modulate their respective catalytic activities. Crystal structures of several SH2 domains have been described, including the one of lck (80). Although SH2s all bind phosphotyrosine residues, adjacent residues can affect the interaction with their substrates. 4) The SH3 domains are about 60 amino acids in length and possess a stretch of poly prolines. These domains are thought to be involved in interaction of proteins with cytoskeletal elements. 5) The kinase domain consists of an ATP binding domain and an autophosphorylation site. 6) Finally, there is a tyrosine residue located at the extreme C-terminal end of the kinase which when phosphorylated, negatively regulates the kinase activity of the protein. This involves an interaction of the phosphorylated tyrosine residue with the SH2 domain of the kinase. The resulting folding of the protein is speculated to block the access of substrates to the catalytic domain. Mutation of this residue to a phenylalanine results in a tyrosine kinase which is constitutively activated and able to transform NIH 3T3 cells.

5.3.1.2 Fyn

Two isoforms of fyn can be expressed resulting from alternative splicing of the seventh exon leading to a 50 amino acid difference at the junction of the SH2 and kinase domains. The fyn T isoform is exclusively expressed in hematopoetic cells whereas the fynB isoform is expressed in neuronal cells. FynT expression is low in immature double positive thymocytes and increases dramatically in mature single positive thymocytes which correlates with the acquisition of the ability of thymocytes to proliferation in response to anti-TcR stimulation (56). Fyn can be co-immunoprecipitated with the TcR\CD3 complex (256). However, the stochiometry of the association is very low (< 2-4% of TcR are associated with fyn) and the use of mild detergents such as digitonin to lyse the cells is required to observe this association. Association of fyn with ζ involves the specific N-terminal domain of fyn and does not require the presence of ARAMs (301). Upon TcR ligation, an increase in tyrosine kinase activity of fyn is observed which occurs within 5 seconds of TcR cross-linking and is maximal at 1 minute (37).

Fyn was also co-immunoprecipitated with PI-linked receptors such as mouse Thy-1, Ly-6 and human CD59, CD48, CD24, CD14, and CD55 or DAF (decay-accelerating factor) (290,299). This association requires two cysteine residues at positions 3 and 6 of fyn and palmitylation of this cysteine motif (274).

Transfection of activated forms of fynT or B (F528) in a T cell hybridoma led to enhanced generation of second messenger such as tyrosine phosphorylation and increase in intracellular calcium, and enhanced IL-2 production induced by anti-TcR stimulation (67).

Only fynT was able to enhance IL-2 production upon stimulation with antigen, thus demonstrating that fyn T has a regulatory function that fyn B lacks in coupling the recognition of antigen to signal transduction. Overexpression of fyn T in transgenic mice led to hypersensitivity to anti-TcR stimulus with respect to protein tyrosine phosphorylation, increase in intracellular calcium, IL-2 production and proliferation (55). Additionally, overexpression of a kinase dead fyn inhibited these activation events (55). On the other hand, mice defective in fyn expression exhibited a normal thymic maturation (13,292). Differences in susceptibility of T cells to stimulation were however noted. Single positive thymocytes were impaired in response to stimulation both at the level of calcium increase and proliferation. The defect in stimulation of peripheral T cells was less pronounced. Splenic T cells had a less pronounced increase in calcium and IL-2 production but had a normal proliferative response to anti-CD3, allogeneic or SEA stimulation. Taken together, these results point to a potentiating role for the tyrosine kinase fyn in T cell activation, which is more pronounced in mature T cells.

5.3.1.2 lck

The lck gene is under the control of two separate promoters resulting in heterogeneity of the 5' untranslated region (331). The proximal promoter lies just 5' of the coding region and directs transcription only in the thymus. The distal promoter is 34 kb upstream of the coding region and directs transcription in both thymocytes and mature peripheral T cells. lck gene transcription was demonstrated to be greatly diminished 6 hours after of stimulation with levels coming back to normal around 24 hours post stimulation (190). This was accompanied by a marked decrease in lck protein present at that time in activated T cells which recovered only 36 hours post stimulation. No functional evidence of the role of this downregulation has been reported up to now.

lck is associated non-covalently with cell surface receptors CD4 and CD8 (250,311). This interaction is mediated by two cysteine residues in the N-terminal specific portion of lck and two cysteine residues in the cytoplasmic domain of CD4 and CD8, and is believed to involve a metal ion (271,272,306) (figure 3). The regulatory function of the association of lck with CD4 will be discussed in details below. lck is also associated with the IL-2 receptor β chain and its kinase activity is increased following stimulation by IL-2 (122,126,197). The function of this association is not well characterized yet but IL-2 can stimulate growth through a mutant receptor that lacks the domain responsible for lck association (122). On the other hand, lck is required to observe activation of the PI3-kinase pathway by IL-2 (297). Similar to fyn, lck is associated with PI-linked cell surface proteins (290,299). The function of these associations is not known although there has

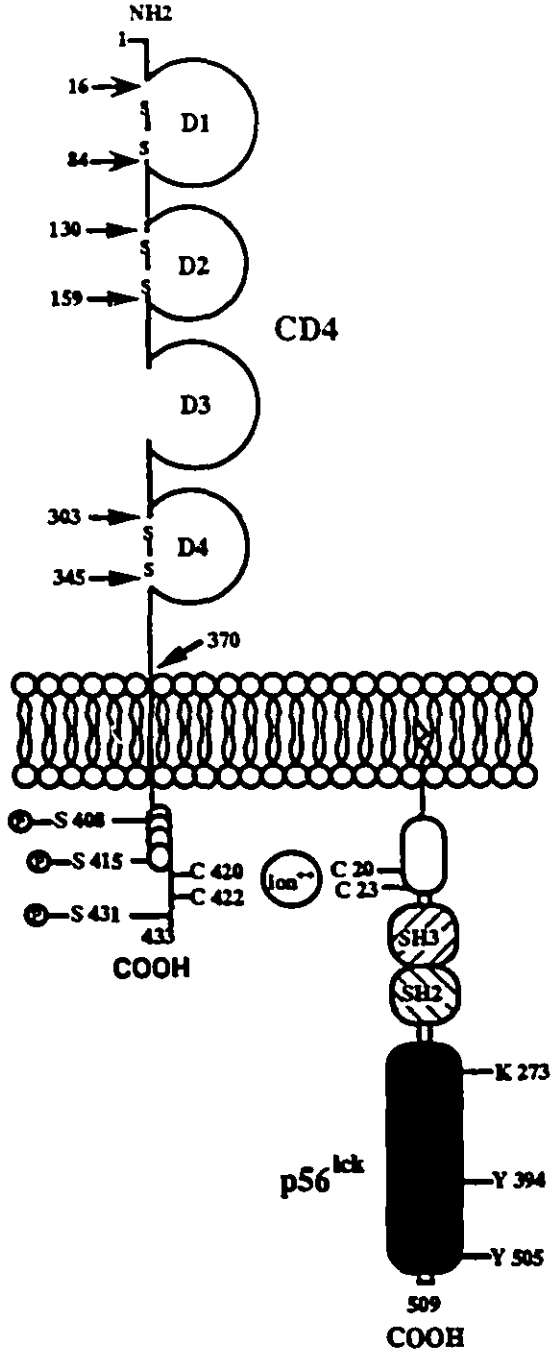

Figure 3 . The domains of the CD4 molecule.

The CD4 molecule consists of four Ig-like extracellular domains, a single transmembrane domain and a short cytoplasmic domain of 38 residues. The membrane proximal cytoplasmic domain can be modeled as an alpha helical structure. This alpha helix together with phosphorylation of the three serine residues at position 408-415-431 are required for endocytosis of CD4 induced by PMA treatment or antigenic stimulation. The association of CD4 with the tyrosine kinase lck is mediated by two cysteine residues of the cytoplasmic domain of CD4 and two cysteine residues of lck, and is believed to require the presence of an ion to stabilize the interaction .

been a report linking anti-Thy induced proliferation with an increase in lck activity (273).

Upon stimulation with either PMA or anti-CD3 Ab, lck becomes phosphorylated on serine residues (180,314). This can be visualized by a retarded migration of lck on a SDS-Page gel. Anti-CD3 treatment induces phosphorylation of about 50% of total lck, occurring via both a PKC-dependent and a PKC-independent pathway (287). These phosphorylations do not seem to modify significantly the activity of lck tyrosine kinase *in vitro*. However, some occur in the SH2 domain and it is tempting to speculate that it may modify the affinity for certain substrates (287,333).

Cross-linking of CD4 with CD4 specific Ab increases the tyrosine kinase activity of the CD4-associated lck, leading to phosphorylation of substrates (181,312). Moreover, stimulation of T cells using anti-CD3 Abs also leads to an increase in lck activity (64). As observed with fyn, this increase occurs within a few seconds after receptor ligation and lasts a few minutes (37). Several tyrosine phosphorylated substrates have been demonstrated to be linked to lck activity. The ζ chain of the TcR complex is phosphorylated following CD4 cross-linking by Ab (312). The effect of this phosphorylation will be discussed in details below. An increase in tyrosine phosphorylation of the GTPase Activating Protein (GAP) and GAP associated p62 was observed in cell lines transfected with an activated form of lck (F505 lck) and lck was found to phosphorylate GAP *in vitro*, thus providing a link between tyrosine kinases and activation of G proteins (8,82). Members of the MAP kinase family are also phosphorylated by lck (86). lck has also been implicated in the regulation of PI metabolism. Indeed, PI-3 and PI-4 kinase activity were co-precipitated with lck (233). Moreover, an increase in phosphorylation of PLC γ was also observed following CD4 cross-linking. In addition, association of PLC γ with lck was observed following TcR stimulation (323). Finally, lck is also involved in cooperation with the oncogene ras to stimulate transcription from the IL-2 promoter (19).

The SH2 domain of lck has been demonstrated to also regulate lck activity (9,104,327). Crystallization studies have shown that lck can dimerize through interaction, between the SH2 and SH3 domains (80). This forms a pocket which binds the phosphorylated tyrosine 505, resulting in an inactive form of lck. The SH2 domain of lck can also mediate the interaction of lck with other proteins. Indeed, it was demonstrated to specifically interact with a number of tyrosine phosphorylated proteins, including the ZAP tyrosine kinase (79,229). Moreover, the importance of the SH2 domain of lck was demonstrated in functional studies where an lck mutated in the SH2 domain could not enhance T cell activation but could transform fibroblast (40,313). These results demonstrated that the SH2 domain of lck regulates the function of lck specifically in T

cells. A role for the SH2 domain of lck was further exemplified in studies which used a chimera between the extracellular domain of CD4 and lck to restore the response to antigen of a CD4-dependent T cell hybridoma (334). In this report, a chimera which was devoid of kinase activity but still possessed an intact SH2 domain could restore the response to antigen whereas a chimera which lacked the SH2 domain but still had kinase activity had a decreased response. A chimera lacking both the SH2 domain and kinase activity could not restore the response of the T cell. Taken together, these experiments point out that the SH2 domain regulates both intramolecular and intermolecular interactions of lck which are important in T cell activation.

Genetic evidence has demonstrated the requirement for lck expression for T cell activation. A mutant of the Jurkat T cell line which lacks expression of lck is deficient in both proximal activation events such as tyrosine phosphorylation and increases in intracellular calcium and distal events such as IL-2 production and induction of CD69 expression (295). Interestingly, re expression of lck in this line restored its ability to respond to TcR stimulus. Another lck negative cell line which is dependent on IL-2 for growth, was impaired in cytotoxic effector function (143). Furthermore, these cells could still grow in an IL-2 dependent manner, although exhibiting some decrease in the growth rate, thus demonstrating that lck is not required for IL-2 stimulation of growth.

The importance of lck was further demonstrated in a T cell hybridoma which was rendered more sensitive to TcR induced activation by the overexpression of an activated form of lck in which tyrosine 505 was mutated to a phenylalanine (2). Again, the enhancing effects were observed both in the generation of second messengers and IL-2 production. Interestingly, myristylation of lck is required to observe this effect suggesting that membrane localization is important in regulation of lck action (3).

lck also plays a major role in thymic maturation of T cells. Mice deficient in lck expression had an early block in thymic maturation resulting in thymic atrophy with only immature thymocytes and almost no T cells in periphery (204). The few T cells that got into the periphery could be activated in vitro to proliferate with mitogens but in vivo, no anti-viral response could be detected in these mice (203,204). These results clearly point out the requirement for lck expression in the generation of an antigen specific response. Overexpression of a dead kinase lck in thymocytes led to severe defect in production of mature T lymphocytes (173). Moreover, a defect in thymic mitogenesis was observed. Conversely, overexpression of wild type or F505 lck induced thymic tumors of immature phenotype (TcR-CD4-CD8-) suggesting that lck sends mitogenic signals (1). In addition, rearrangement of the TcRβ locus was severely impaired in these mice (1,12). Interestingly, overexpression of lck which does not associate with CD4 also prevented

rearrangement of the β locus demonstrating that lck can act independently of its association with CD4 and CD8 (172).

5.3.2 The ZAP/syk Tyrosine Kinases

The ZAP tyrosine kinase was first identified biochemically as a 70 KDa tyrosine phosphorylated protein which becomes associated with phosphorylated ζ upon TcR stimulation, thus its name "zeta associated protein" (42,43). The ZAP kinase is a member of a new tyrosine kinase family of which syk is the prototype. These cytosolic kinases possess 2 tandem SH2 domains and no SH3, a tyrosine kinase domain and no negative tyrosine residue (figure 2).

Both syk and ZAP are expressed in T cells although their expression is differentially regulated (45). Indeed, ZAP is expressed only in T cells and natural killer cells. Expression of ZAP is detected at all stages of maturation and in peripheral T cells. Syk can be found in a number of hematopoetic cells, such as thymocytes, B cells and myeloid cells. Although syk is expressed in high levels in thymocytes, it is greatly down regulated in peripheral T cells. This difference in expression suggests they play different role at different stages of T cell maturation. Unlike lck, there is no downregulation of expression of ZAP or syk following activation.

The functional heterogeneity of these two kinases is further supported by biochemical data which demonstrates differential requirement for activation of these kinases (149). Indeed, chimeric molecules consisting of the extracellular domain of the CD16 surface antigen and tyrosine kinases have demonstrated that cross-linking of the syk chimera activated T cell cytolytic pathway, whereas activation through the ZAP chimera required co-cross-linking of lck or fyn chimeras. Cross-linking of either alone however was sufficient to induce an increase in intracellular calcium.

Genetic evidence for the importance of ZAP in T cell maturation and activation came from studies which demonstrated that human SCID patients had a defect in ZAP expression (14,44,81). These patients exhibited no CD8 positive T cells in periphery but CD4 positive T cells were present. However, stimulation of these CD4 positive T cells was almost totally impaired. The role of syk in T cell activation is less characterized. There has been some reports though that suggest that syk can activate lck tyrosine kinase activity in vitro (59).

Both the ε and ζ chains of the TcR complex can associate with ZAP (294,321). These interactions are mediated through the ARAM sequences of the ζ and ε chains. The tyrosine residues located in this motif become phosphorylated upon stimulation and are anchor residues for second messengers such as the ZAP tyrosine kinase. Experiments

using synthetic peptides that includes the ARAM sequence have demonstrated that the presence of both phosphorylated tyrosine residues are essential for the motif to be functional for association with ZAP (134,322). Conversely, the presence of the two tandem SH2 domains on ZAP is required for this association (134,322). Moreover, the association of ZAP SH2 domains with ζ was demonstrated to prevent dephosphorylation of the ζ chain. ZAP tyrosine kinase activity is not required for association of the phosphorylated ZAP to the ζ chain (134). lck expression and tyrosine kinase activity are required for these events to occur since no ZAP association with ζ induced fcllowing TcR ligation was observed in an lck negative T cell line (295). In reconstitution experiments using the Cos system, co expression of either lck or fyn was required to obtain tyrosine phosphorylation of ZAP and its association with the ζ chain (117,134). Thus, the first event following T cell activation would be phosphorylation of the tyrosine residues of the ARAMs by lck and/or fyn, followed by association of ZAP with the ARAMs and subsequent tyrosine phosphorylation of the associated ZAP (figure 4).

Recently, it was reported that the SH2 domain of lck can bind tyrosine phosphorylated ZAP (79,300). Moreover, the authors showed that the interaction between CD4 and the TcR was mediated through the interaction of the CD4-associated lck with the ζ-associated ZAP. These results suggest that the interaction between ZAP and lck serves as an amplifier of the initial signal by recruiting in the TcR complex proximity other molecules involved in signal transduction (figure 4).

Finally, two pools of phosphorylated ZAP were identified: one associated with ζ and the other associated with a protein of 120 KDa (326). This report suggests that the mode of activation of ZAP is more complex than the current model. Further studies are required to understand the interplay and activation mechanisms of the tyrosine kinases involved in the initiation of T cell activation.

5.4 Regulation of fyn and lck activity

5.4.1 csk

As mentioned, src-related tyrosine kinases are negatively regulated by phosphorylation of their C-terminal tyrosine residue (Y505 for lck, Y528 for fyn). The tyrosine kinase csk can phosphorylate specifically these tyrosines in vitro leading to a concomitant decrease in tyrosine kinase activity of fyn or lck (27). csk possesses an SH2 domain, an SH3 domain and a tyrosine kinase domain. It lacks however a positive autophosphorylation site, a negative phosphorylation site and a myristylation signal (210).

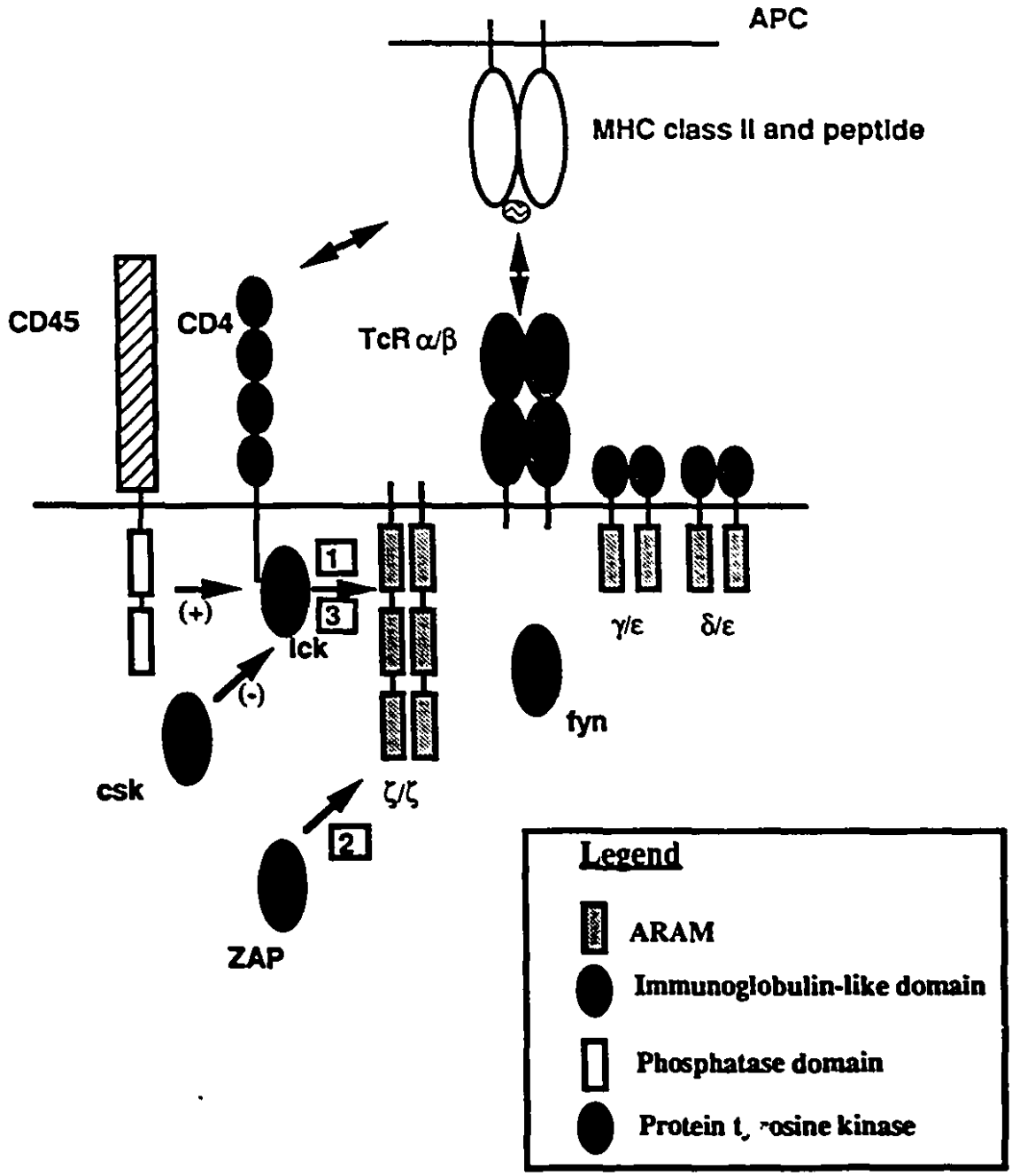

Figure 4. Early events of T cell activation.

T cells recognize the peptide/MHC complex through their specific TcR. Interaction between CD4 and MHC class II molecules augments the response of T cells to antigenic stimulation. The first detectable event following antigen recognition is tyrosine phosphorylation. The ARAMs are tyrosine phsophorylated in an lck/fyn dependent fashion (1). This allows the association of ZAP with the ARAMs and its phosphorylation (2). ZAP can also induce the association of the TcR and CD4 through its interaction with the CD4-associated lck SH2 domain (3). CD45 and csk are involved in positive and negative regulation of fyn and lck activity. Association of CD45 with CD4 and the TcR was also reported.

Several tyrosine kinases have been cloned recently which share the same structure and substrate specificity as csk and have a more restricted expression pattern, thus defining a new family of tyrosine kinases (49,147).

How csk kinase activity is regulated is not known at the present time. The potential role of csk in regulating T cell activation was demonstrated in experiments which used a T cell hybridoma overexpressing the csk tyrosine kinase (50). This hybridoma was unresponsive to antigen or anti-TcR stimulation in that there were no increase in tyrosine phosphorylation or IL-2 produced. Interestingly, this defect could be rescued by co-transfection of an activated form (F528) of fyn. These results strongly suggest that csk or a csk like tyrosine kinase negatively regulates T cell activation by phosphorylating C-terminal tyrosine residues of fyn and lck thus inhibiting the tyrosine kinase activities of these src-related tyrosine kinases.

Mice bearing a null mutation in the csk gene die while still a fetus. Investigation of the role of csk in T cell maturation was done by the generation of a chimeric mice obtained by injection of ES csk null cells into RAG-2 null blastocysts which express no TcR due to lack of rearrangement of the TcR genes (110). The amounts of csk negative cells was high enough only to be analyzed in the embryo before day 17. The results show that the thymus can be colonized by early precursors CD4loCD8-TcR- but that these thymocytes do not differentiate and are overcome by the RAG-2 null thymocytes before birth. This suggests that csk plays a role in thymocytes differentiation independently of regulation of lck and fyn because the block in maturation in csk null thymocytes is earlier than the ones observed in lck and fyn null thymocytes.

5.4.2 CD45

CD45 is a transmembrane protein expressed exclusively on all cells of the hematopoetic lineage but not on platelets and erythrocytes. CD45 is highly expressed and can comprise up to 10% of cell surface molecules on T cells. The 705 amino acids cytoplasmic domain of CD45 contains two tandem tyrosine phosphatase domains. At least eight isotypes of CD45 (180-235 KDa) can be expressed which arise from alternative splicing resulting from the use of different exons 4,5,6 (or A,B,C) coding for the extracellular domain (reviewed in 304). Isoforms have been defined by Abs specific for sequences encoded by the different exons; exon-4 containing CD45 is the CD45RA isoform, exon-5 is the CD45RB and exon-6 is the CD45RC isoform of CD45. Expression of these isoforms is regulated both at the level of tissue specificity and development. One T cell can expressed multiple isoforms of CD45. Switch of expression of CD45 isoforms from high mwt isoforms to low mwt isoforms are observed when naive

cells become activated and memory cells. Expression of CD45 isoforms can also vary depending on the cytokines produced by the activated cells (TH1 and TH2 cytokine profiles) (169).

Studies have shown that anti-CD45 Ab can either upregulate or downregulate T cell activation. Cross-linking of CD45 with CD4 enhances anti-TcR stimulation whereas cross-linking of CD45 with the TcR inhibits stimulation (107,168). This inhibition can be reversed by phosphatase inhibitors suggesting that modulation of phosphatase activity is implicated in this phenomenon.

The confirmation of the importance of the phosphatase activity of CD45 in T cell activation came from studies using mutant cell lines lacking expression of CD45. These cell lines cannot produce IL-2 following TcR stimulation; this is accompanied by a complete abrogation of tyrosine phosphorylation, including phosphorylation and activation of PLC γ (151,232), and a disturbed calcium homeostasis (316). Transfection of CD45 restored their ability to be stimulated.

Both lck and fyn were demonstrated to be hyperphosphorylated at their C terminal negative regulatory site in CD45 negative cell lines (191,218). Furthermore, this phosphorylation was accompanied with a decrease in autophosphorylation activity in vitro. These results strongly suggested that CD45 phosphatase activity is required to dephosphorylate and activate lck and fyn. Indeed, CD45 can be co-immunoprecipitated with lck independently of its association with CD4 or CD8 (249,265). In addition, in vitro studies have clearly demonstrated that CD45 does dephosphorylate lck and fyn , leading to an increase in their kinase activity (129,208,209,278,280). One report however does not observe a decrease in activity but rather an increase in tyrosine kinase activity in CD45 negative cell lines (38). Consistent with this report, cross-linking of CD45 with CD4 leads to a decrease in lck activity in vitro (219). These contradictory results may be explained by the presence or abundance of other proteins regulating lck activity in the different cell lines used.

The importance of CD45 in T cell development was observed in mice deficient for exon 6 of the CD45 gene (146). A block in thymocyte maturation at the transition between double positive to mature single positive was observed. Consequently, there was a reduced number of T cells in the periphery and their response to mitogens was significantly reduced. These observations are in agreement with what was observed in cell lines on the importance of CD45 in T cell activation. More recently, cross-linking of CD45 in vivo by Ab, resulted in an increase in lck activity associated with CD4 in double positive thymocytes (26). This inhibited maturation of T cells into single positives by

inhibition of positive selection. These results further support the importance of CD45 in thymic maturation.

The fact that CD45 is expressed at high levels on T cells and that its activity is crucial in T cell activation suggests that the activity of CD45 must be finely regulated. One of the models is that CD45 would be regulated through interactions with other cell surface molecules. Different isoforms of CD45 would interact with different ligands. The extracellular domain of CD45 is heavily glycosylated. Both N- and O-linked sugars are present and glycosylation levels vary between isoforms which may regulate the interaction of CD45 with other molecules. To date, only one ligand expressed on the APC has been identified, CD22. On the other hand, CD45 has been co-precipitated with a number of T cell surface molecules notably, CD4 and CD8, CD2, Thy-1, and the TcR complex (74,200,266,317). But no modulation of the phosphatase activity of CD45 by association with these molecules has been reported. However, chimeric molecules lacking the extracellular and transmembrane domains of CD45, restored completely stimulation of CD45 negative cells (127,318). These results suggest that the expression of the extracellular domain of CD45 is not required to couple the TcR to the signalling machinery. Reconstitution was also achieved with a chimera between the EGFR and the cytoplasmic domain of CD45 (72). Interestingly, EGF stimulation of the chimera abrogated the signaling potential of the chimera. These results suggest that the phosphatase activity of CD45 may be negatively regulated in a way similar to transmembrane tyrosine kinase, i.e. through oligomerization of the active site.

CD45 can also be phosphorylated both on serine and tyrosine residues. Phosphorylation on serine residues is constitutive although IL-2 can induce stronger phosphorylation (309). Moreover, ionomycin treatment of thymocytes and certain cell lines resulted in dephosphorylation of CD45 on serine residues leading to a decrease in phosphatase activity of CD45 (220). These results suggested that CD45 activity can be regulated by a calcium dependent pathway. Tyrosine phosphorylation of CD45 can be observed transiently following TcR stimulation (293). Also, csk can phosphorylate CD45 on a tyrosine residue located in one of the phosphatase domain *in vitro* (17). This phosphorylation induced lck association through its SH2 domain with CD45 *in vitro* and correlated with an increase in tyrosine phosphatase activity of CD45. More work is required to characterize the kinases and the mechanisms of regulation involved in phosphorylation of CD45, and the relevance of these phosphorylations in regulating its activity.

6. THE CD4 MOLECULE

6.1 Structure and expression

The CD4 molecule is a 55 KDa glycoprotein which is a member of the Ig super family. The CD4 gene expression is under the control of a promoter and an enhancer which does not discriminate between the T cell lineages (CD4+ and CD8+) (29,260). The differential expression is rather regulated by a cell type specific silencer (261). The nucleotide sequence of the CD4 gene is highly conserved between mouse and human with homology of 55% for the extracellular domain and of 75% for the cytoplasmic domain. Indeed, several pieces of evidence seem to support, including chapter 3 of this thesis, that human CD4 can function properly in murine T cells. The CD4 molecule consists of four Ig-like extracellular domains (D1 to D4), a single transmembrane domain and a short cytoplasmic domain of 38 residues which mediates the interaction with lck (figure 3). The human protein has two potential glycosylation sites whereas the murine CD4 has four. The CD4 molecule is mainly expressed on a subsets of T lymphocytes (239) but can be detected at low levels on monocytes, granulocytes, eosinophils and dendritic cells (184).

Between D2 and D3 , a stretch of five amino acids confers flexibility to the CD4 molecule (34,68,158). The effect of this hinge region was indeed observed in crystals of the CD4 molecules which revealed an heterogeneity of the relative position of D1-D2 to D3-D4. Because of this, a good resolution of crystals of CD4 could only be obtained by cutting the molecules in half, D1-D2 (252,320) and D3-D4 (34), and crystallizing independently each half.

The analysis of the crystals of D1D2 and D3D4 revealed a striking homology between these domains, suggesting a possible duplication of one pair to form the whole CD4 molecule. Moreover, the crystal structure of D1D2 is almost superposable on D3D4 but the relative position of D3 to D4 is moved 30 degrees.

Like Igs, each domain is composed of two β sheets linked by di-sulfide bridges. D1 and D3 are composed of nine anti-parallel β strands (A,B,C,C',C",D,E,F,G,). The protruding loops between strands B and C, C" and C', F and G, have been designed respectively CDR1, CDR2, and CDR3 in homology with Igs. Like Ig, these loops were found to be involved in interaction of CD4 with its ligands as will be discussed below.

Analysis of crystals of the entire CD4 molecule revealed the presence of tetramers of CD4. However, this property was not observed in crystals of D1D2 and D3D4, and no biochemical evidence for tetramers has been reported yet. This suggests that this complex may either exist with whole CD4 molecules or be an artifact of crystallization.

6.2 Interaction with MHC class II molecules

An interaction between CD4 and MHC class II molecules was first suspected following the observation of the strict correlation of recognition of peptide/MHC class II by CD4 + T cells and peptide/MHC class I by CD8+ T cells (189,296,330). Transfection of CD4 into CD4 negative T cell hybridomas greatly increased the response of the hybridomas to antigen stimulation (100,108,159,282). Moreover, the interaction between CD4 and MHC class II in both a lymphoid and non-lymphoid context induced aggregation between the cells expressing these molecules (78,159,160). Thus, the CD4/MHC class II interaction was first described as an adhesion interaction increasing the avidity of the T cell for the APC.

Mutagenesis studies have demonstrated that the interaction between CD4 and MHC class II involves residues located in three exposed loops (including the CDR1 and CDR3) of the D1 and D2 of CD4 all located on one face of the CD4 molecule (32,52,53,92,202). Contradictory results have been reported on the implication of the CDR2 of CD4 in interaction with class II. Both lymphoid and non-lymphoid systems have been used to characterize this interaction. This was done either in the context of antigenic recognition or simply in adhesion assays independently of TcR recognition. Since there are reports of association between CD4 and the TcR (see below), it is possible that CD4 does not interact with MHC class II molecules in the same way whether the TcR recognizes antigen or not, thus explaining the apparent contradictory results.

The association of lck with CD4 can modulate the interaction of CD4 with MHC class II and lck activity can also influence interactions involving other adhesion molecules (LFA-1) (303). Using a non lymphoid system, it was demonstrated that association of CD4 with lck increases the binding of CHO transfected with CD4 to class II molecules on B cells (145). This enhancement of adhesion was accompanied by an increased association of the CD4/lck complex with the cytoskeleton and formation of adherens-type junctions between the cells.

The crystal structure of HLA-DR MHC class II molecules was recently resolved and revealed the presence of dimers of MHC class II molecules (35). More recently, biochemical and functional data also supported the presence of dimers of class II molecules (262). This could also influence the way class II molecules interact with CD4 molecules. Interaction of CD4 with dimers could indeed require other residues on CD4 than those implicated during interaction with monomers of MHC class II molecules. More work however is required to characterize the formation of these dimers and their exact function.

This leads us to another interesting point: does CD4 interact with the same class II as the TcR? As mentioned, several studies have demonstrated that CD4 transfected in nonlymphoid cells can interact with MHC class II molecules on B cells leading to aggregation of the two cell types (78). These studies suggested that CD4 and MHC class II can interact independently of the TcR, although this interaction seemed to require higher levels of expression of CD4 than what is usually found in physiological conditions. CD4 was also demonstrated to interact with MHC class II molecules independently of the TcR when expressed in a T cell hybridoma whose TcR recognize MHC class I D^d (108). In this system, CD4 interaction with MHC class II lead to an enhancement of the stimulation of T cell which was measured by IL-2 production. But other experiments, including those reported in chapter two of this thesis, seem to suggest that in more physiological conditions the CD4 must interact with the same MHC class II than the TcR does. This would be required to bring in close contact the CD4 associated lck and the TcR signalling complex. In addition, CD4 and the TcR interact with each other during the antigen recognition process (see below). In thymic selection, it was demonstrated that CD8 must interact with the same MHC class I than the TcR for positive selection to occur (131). More recently, CD8 was demonstrated to regulate the TcR-antigen interaction by interacting with the same class I molecule than the TcR (179). There is however no experiment addressing this question reported yet for the positive selection of CD4 thymocytes.

6.3 Interaction with the TcR complex

Co-aggregation of the TcR and CD4 was first described in immunofluorescence experiments demonstrating that recognition of the proper peptide/MHC class II complex by the TcR induces the co-capping of the TcR and CD4 (155,156). This is characterized by a clustering of these molecules polarized at the site of interaction of the T cell with the APC. MHC class II molecules on the APC that are specifically recognized by the TcR also form a cap at the recognition site. The LFA-1 molecule which is implicated in the adhesion of the T cell with the APC also caps at the contact site (153). The capping of these cell surface molecules is accompanied by a reorganization of the microtubule organizing center (MTOC) now facing the contact site between the two cells, and an accumulation of the cytoskeletal protein talin under the cytoplasmic membrane at the contact site (157).

The co-capping of CD4 and TcR can also be induced using anti-TcR Abs (154,254). Interestingly, a correlation can be drawn between the ability of anti-TcR Abs to induce co-capping of CD4 and the potency of the anti-TcR Abs to induce

phosphorylation of the ζ chain of the TcR complex (75,245). This supports the hypothesis that CD4 and the TcR synergize and recognize the same MHC (co receptor function of CD4). The mechanistical basis for this is not determined yet. A conformational change induced by anti-TcR Abs could lead to association of CD4 with the TcR. Conversely, TcR ligation could send intracellular signals to CD4. Interestingly, TcR ligation was demonstrated to trigger changes in the affinity of CD8 for class I molecules (216). This was not reported for CD4 however.

Anti-TcR Abs or antigen were also demonstrated to induce internalization of both the TcR and the CD4 molecules (10,254,329). Interestingly, anti-CD4 Abs blocked this co-modulation (243). Moreover, this co-modulation was observed only in activated T cells and not resting T cells .

Resonance transfer experiment also demonstrated a physical interaction between the TcR and CD4. Interestingly, this required lck association with CD4 (54). Finally, biochemical data clearly identified an association between CD4 and the TcR complex. Indeed, the CD4/lck complex could be immunoprecipitated with the ζ, ε and δ chains of the TcR complex (36).

Why and how the TcR and CD4 interact has been the subject of a number of studies and is not completely characterized yet. This interaction and the fact that CD4 is associated with lck which is required for T cell activation, suggest that CD4 can generate signals regulating early events of T cell activation.

6.4 The signaling function of CD4

The first evidence that CD4 could indeed generate signals regulating T cell activation came from experiments whereby MHC independent stimulation of T cells could be inhibited by anti-CD4 Abs (248,302). More recently, crosslinking of CD4 independently of the TcR was demonstrated to lead to cell death by apoptosis (20,211). It was then proposed that asynchronous stimulation through CD4 and TcR was sending a negative signal. On the contrary, co-aggregation of CD4 with the TcR resulted in an enhanced stimulation of the T cell (221). The discovery that CD4 was associated with a tyrosine kinase, lck, and that cross-linking of CD4 with Ab increased the activity of lck led to a large number of studies on the role of the CD4/lck association in regulating T cell activation events.

Using a T cell hybridoma which requires CD4 expression to produce IL-2 in response to stimulation by antigen, Glaichenhaus showed that association of lck with CD4 was required to restore the response to antigen (105). These results and results presented in chapter 2 of this thesis, suggested that CD4 must bring lck in close contact with the

TcR complex in order for T cell activation to initiate. Indeed, lck association with CD4 was shown to be required for enhanced stimulation following co-cross-linking using Ab of the TcR and CD4 (51,76). This stimulation leads to an increase in recruitment of ZAP association to ζ which is dependent on lck activity. Moreover, a report suggested that expression of the ζ chain was required to observe an enhancement of activation following the co-crosslinking of TcR and CD4 (257). As mentioned, the association of CD4 with lck was found to be required to observe CD4 interaction with the TcR (54). Taken together, these reports thus suggest that the physical interaction between the TcR and CD4 is mediated intracellularly by lck and is required to initiate T cell activation.

Recent studies suggest that the extracellular domain of CD4 may regulate T cell activation independently of its association with lck (315,338). This could be a direct interaction of the extracellular domain of CD4 with the TcR to augment the avidity of the TcR for the peptide/MHC complex, or an interaction with another T cell surface molecule. Interestingly, CD4 was shown to be associated with CD45, a key regulatory molecule for T cell activation by both biochemical and immunofluorescence approaches (74,200) in an isoform specific manner. An isoform specific interaction of CD45 with external domains of other cell surface markers of T cells such as CD4, could modulate T cell activation. Further studies are required to assess specifically if these interactions modulate the phosphatase activity of CD45.

6.5 Internalization of CD4

Upon PMA or anti-CD3 treatment, CD4 becomes phosphorylated on three serine residues in its cytoplasmic domain (28,130,230,275). In human CD4, the three residues are located at positions 408, 415 and 431 (figure 3). Phosphorylation is the strongest (80%) on serine 408 and the effect of this phosphorylation on internalization is also the strongest as mutation of this residue rendered CD4 almost totally resistant to PMA (275). This phosphorylation of CD4 induces its internalization via clathrin-coated pits and degradation in the lysosomes (228,276). Studies have shown that lck association to CD4 prevents internalization of CD4 (227). Consequently, PMA-induced phosphorylation of CD4 leads to dissociation of lck from CD4, thus rendering it more susceptible to internalization (228,283). More recently, the group of Strominger has proposed that a putative alpha helical structure in the cytoplasmic domain of CD4 was also required to target CD4 to degradation (figure 3) (276). The role of this internalization of CD4 is not well characterized yet. There was however a report demonstrating that a mutant in which all three serine residues were changed for alanine residues could not restore a CD4

dependent antigenic response of a T cell hybridoma, thus suggesting that dissociation of lck from CD4 may be required for lck to phosphorylate its substrates (105).

7. THE HUMAN IMMUNODEFICIENCY VIRUS (HIV)

CD4 is the receptor for the HIV. The viral envelope protein gp120 binds to CD4 and viral entry ensues by fusion of membranes. As will be discussed below and presented in the following chapters, gp120 interaction with CD4 can modulate the CD4 function , altering early activation events and HIV replication. Moreover, several viral proteins can directly downregulate CD4 expression. A brief overview of the mechanism of disease and the viral cycle of HIV will be given followed by a more detailed review of the effect of HIV proteins on expression of CD4 and the generation of signals through CD4.

7.1 Mechanism of disease

Infection with HIV ultimately results in the Acquired Immunodeficiency Syndrome (AIDS) (reviewed in 225). This disease is defined by a gradual loss of CD4 T lymphocytes paralleled by an increasing immune dysfunction which is characterized by the occurrence of opportunistic diseases. The disease progression is characterized by a clinical latency period which lasts on average 8-10 years, but that can be as short as a few months or longer than 15 years. However, during that period, there is a tremendous amount of viral replication going on in lymph nodes (83,224). Studies on the dynamics of HIV infection demonstrate a vigorous immune response against HIV is occurring implicating both cytotoxic T cells and the humoral response. The course of the disease is governed by a balance between viral replication and viral clearance by the immune system (125,325). Unfortunately, the virus ultimately wins and the infected host dies.

The mechanism of immune deficiency is not completely understood yet. Patients show a decreased response to recall antigen stimulation in vitro (89,114,162). Infection of T cells with HIV has been demonstrated to directly impair stimulation of T cells by anti-CD3/TcR Ab (175). In addition, isolated viral proteins such as gp120 and Nef (see below) can on their own affect T cell activation events. The severe loss of CD4 T cells is believed to contribute greatly to the immune dysfunction as these cells play a pivotal regulatory role in the course of immune responses.

Several mechanisms have been implicated in the loss of CD4 T cells in infected patients (reviewed in 195). The decrease in CD4 T cells can not be totally accounted for

by the cytopathic effect of infection since only a small percentage of T cells are infected. Direct cytolysis of infected cells by HIV specific cytotoxic T cells is believed to contribute to this loss. Moreover, apoptosis has been observed in PBLs isolated from infected patients (7,111,166,194). The exact cause of apoptosis is not well characterized yet but exposure of T cells to the viral proteins gp120 and tat can result in apoptosis of T cells *in vitro* (328). Autoimmunity has also been proposed as a model for CD4 T cell depletion. Finally, it was proposed that a yet unidentified HIV encoded superantigen could selectively lead to the deletion of specific T cell subgroups (165,237).

7.2 Structure and genes

The HIV is a retrovirus belonging to the lentivirus family (reviewed in 308). The virion has a characteristic cylindrical core of about 110 nm in diameter formed by the p24(p25) Gag protein (figure 5). Contained in the core are two copies of 35S single-stranded genomic RNA with the polarity of mRNA which are closely associated with the viral polymerase reverse transcriptase and the nucleocapsid (NC) proteins (p9, p6). The inner membrane is composed of the matrix protein (p17) which is myristylated and is required for proper assembly of the virion. The outer membrane has 72 knobs of trimers or tetramers of the envelope protein (env) which is composed of a surface gp120 non-covalently associated with a transmembrane gp41.

Like other known retroviruses, the genome of HIV is composed of env, gag and pol genes flanked by long terminal repeats (LTR) (figure 5). The gag gene encodes viral structural proteins : the matrix p17 and p24, and the capsid protein p6/9. The pol gene encodes for three enzymes: protease, reverse transcriptase and integrase. The env gene encodes for gp160 precursor which is further cleaved into gp120 and gp41. At least 6 other proteins are encoded by the HIV genome. These includes the regulatory proteins tat, rev, vif, vpr, vpu, and Nef.

7.3 viral life cycle

The receptor for the HIV is the CD4 molecule (62,148,161,259). The viral env glycoprotein gp120 binds to CD4 and viral entry ensues by fusion of the two membranes(291). Recently, a co-factor aiding in viral entry was identified as the peptidyl transferase CD26(39). However, this result is still controversial (167). The affinity constant for binding of gp120 to CD4 is in the order of 10^{-9} M and is not affected by deletion of the cytoplasmic domain of CD4. Moreover, viral entry is very efficient in

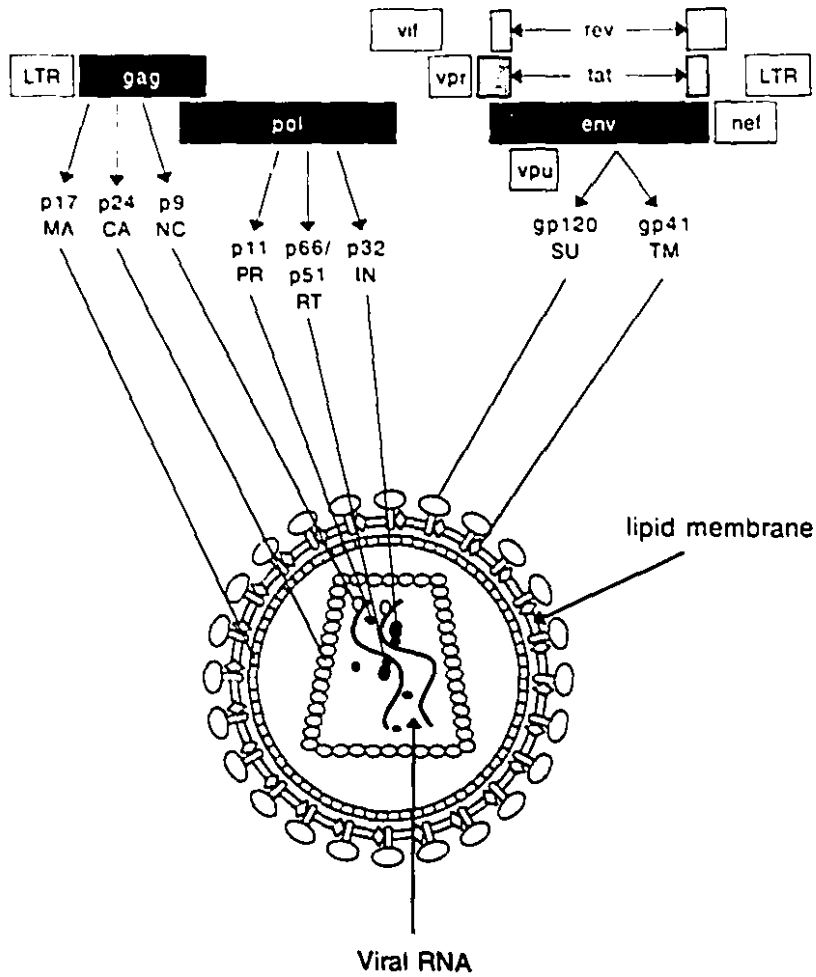

Figure 5

HIV structure and gene organization.

cells expressing a CD4 molecule lacking its cytoplasmic domain, suggesting that this event does not require endocytosis of CD4 mediated through the serine endocytosis motif (24,73,183). However, viral binding to CD4 was demonstrated to induce phosphorylation of CD4 (91).

The amino acids of the extracellular domain of CD4 implicated in binding of gp120 were identified as being part of the CDR2 loop of the first Ig-like domain (15,16,53,92,159,160,201,231). Deletion of residues 43-52 resulted in total abrogation of gp120 binding to CD4. Residues in the third domain of CD4 have also been implicated in viral entry events post-binding of gp120 (305). Interestingly, residues of CD4 implicated in binding of gp120 are dissociable from residues implicated in the interaction of CD4 with MHC class II molecules (92,159,160). The residues on gp120 implicated in binding to CD4 were also identified. Deletion of 12 residues (389-407) in the relatively conserved C-terminal region spanning residues 397-439 completely abrogated the binding of the mutant gp120 to CD4 (164).

Once inside the cytoplasm, the viral genome is reverse transcribed into double-stranded proviral DNA by the RNA/DNA-dependent DNA polymerase and ribonuclease H activities of the viral reverse transcriptase (reviewed in 61). The viral DNA can exist in two forms, either circular or linear. It is then incorporated into the host chromosomal DNA in a process which requires the viral endonuclease encoded by the pol gene. Successful integration of viral DNA into host chromosomal DNA in T cells requires activation and proliferation of the infected cell. Reverse transcription can occur in resting T cells, but it is not complete and results in a partial proviral DNA which can remain in the cytoplasm for days to weeks. In monocytes and macrophages however, productive infection can occur without concomitant proliferation, suggesting that the control of HIV infection varies between different cell types.

Once integrated, the provirus can either be silent (latent) or lead to productive infection. Expression of viral genes is under the control of both host and viral factors. Transcription is mediated by the cellular RNA polymerase II. The promoter element contains a TATA box and Sp1 sites which increase the rate of initiation of transcription. The enhancer of the HIV promoter contains sites for NF-κB and NF-AT and AP-1. There is also a negative regulatory element (NRE) which negatively regulates transcription. The fact that cellular transcription factors such as NF-κB which are required for HIV promoter activity are only found in activated T cells further demonstrate the requirement for activation of the infected T cell to get a productive infection.

Two classes of transcripts can be defined based on their temporal appearance in the viral cycle. Early transcripts consist of fully spliced 2 kb class of viral mRNA that

encode for the regulatory proteins tat, rev and Nef. The viral tat is a transactivator that dramatically increases transcription from the HIV LTR. A functional tat is required for viral replication *in vitro*. Also essential is a copy of the cis-acting viral sequence responsive to tat, the TAR element. The regulatory protein rev controls the appearance of late class mRNAs which consists of the unspliced (9 kb) transcripts which code for the gag-pol, and singly spliced (4 kb) transcripts that code for structural proteins. Rev deficient virus is replication defective with no late mRNAs produced leading to the absence of structural proteins. The appearance of late mRNAs correlates with a decrease in fully spliced mRNAs. Thus, rev negatively regulates its expression and establishes a balance between regulatory and structural proteins being produced. The function of Nef will be discussed in details below.

Viral assembly and release at the cell membrane requires a specific interaction between the viral genomic RNA with the gag-derived nucleocapsid (NC) protein. gag and pol proteins are incorporated into the virions in the form of precursor proteins that are proteatically cleaved by the viral protease during or after budding of the mature viral particles.

7.4 Downregulation of CD4 expression by viral proteins: vpu, gp120 and Nef

CD4 cell surface expression is dramatically decreased in HIV infected cells (reviewed in 102). A decrease in CD4 expression is speculated to prevent viral superinfection. A decrease in CD4 mRNA has been reported to occur in infected cells but the mechanism by which this occurs is not characterized (101,128,255). However, three viral proteins have been implicated in downregulation of the CD4 protein: gp120, vpu and Nef.

gp160 downregulates CD4 expression by associating with newly synthesized CD4 molecules in the ER, thus preventing egress of the CD4/gp160 complex to the cell surface (30,60,136). Moreover, exposure to gp120 of T cell clones leads to endocytosis of cell surface CD4 (41). In addition, the vpu protein ,in conjunction with gp160, induces rapid degradation of CD4 in the ER, leading consequently to a decrease in cell surface expression of CD4 (332). In order to observe this effect, CD4 must be trapped in the ER by gp160 or treatment of cells with brefeldin A that blocks transport of newly synthesized protein out of the ER. We have demonstrated that a putative α helical structure in the cytoplasmic domain of CD4 is required to observe degradation of CD4 induced by vpu (335). Furthermore, this event does not require either lck association with CD4 or phosphorylation of the serine residues in the cytoplasmic tail of CD4. A role of this

degradation of CD4 is to facilitate the release of virions by disrupting gp160/CD4 complex.

The effect of Nef on CD4 expression will be discussed in details below.

7.5 The effect of gp120 on T cells

As was discussed above, the CD4 molecule plays a critical role in early T cell activation events. Consequently, gp120 was demonstrated to generate signals through CD4 and modulate T cell activation. In resting PBLs, oligomeric forms of gp120 can induce an increase in lck activity, in tyrosine phosphorylation of several substrates including PLCγ and in intracellular calcium (106,142,152,286). Moreover, this treatment induces progression in the G1 phase of the cell cycle as determined by the characteristic increase in size of treated cells. Cell surface expression of several activation markers such as HLA-DR and the IL-2 receptor α are also induced. Interestingly, the resulting T cells are anergic to TcR induced stimulation (48,106,186,199). gp120 expressed on the surface of fibroblasts can also inhibit the response of both T cell clones and PBLs to anti-TcR stimulation thus suggesting that in vivo gp120 expressed on the surface of infected cells can act on adjacent CD4 T cells and modulate their activation (267). In addition, T cells treated with gp120 are programmed to undergo apoptosis following TcR ligation (21). This observation is interesting since apoptosis is observed in infected patients and is speculated to be one of the mechanisms by which the CD4 T cells are depleted in HIV infected patients.

gp120 binding to CD4 was also demonstrated to activate lck activity in T cell lines and lead to impaired T cell activation as measured by IL-2 production (124,222). Moreover, this treatment can lead to impaired binding activities of several transcription factors involved in transcription from the IL-2 promoter such as NF-AT, NF-κB and AP-1 (135). These transcription factors are also involved in regulating HIV LTR driven transcription. Thus exposition of infected T cells to gp120 could also modulate HIV replication as will be discussed in chapter 5.

Antigen stimulation of an human CD4 positive T cell clone was also found to be impaired following exposure to gp120. This was accompanied by down modulation of cell surface expression of CD4 and dissociation of lck from CD4 (41). In this model, impairment of T cell activation would be caused by a lack of co-stimulatory activity of the CD4/lck complex.

Altogether, these experiments clearly show that gp120 can contribute to the immune dysfunction which is observed in HIV infected individuals.

7.6 Nef

7.6.1 Structure

The *nef* gene is located in the 3' LTR open reading frame (ORF) of the HIV genome (6,115). The Nef gene product is a 27 KDa myristylated protein (6,93) which is expressed abundantly and early in the viral cycle (90,206). Indeed, up to 80% of early mRNA can code for Nef (244). Nef is predominantly associated with the cytoplasmic compartment or partly associated with membranes, although there have been reports of nuclear localization (93,207).

The characterization of the effect of Nef on HIV replication has been the subject of a vast number of studies, almost all of which seem to contradict each other. Indeed, both a positive and a negative effect of Nef on HIV replication in vitro have been reported. There is one observation which is generally agreed upon: Nef induces downregulation of cell surface expression of CD4 (11,98,99,188,241).

7.6.2 Downregulation of CD4 expression

Downregulation of CD4 expression can be induced by the presence of Nef isolated from a large number of clinical HIV isolates (11,98,188,298). Myristylation of Nef was found to be required to observe this effect and for the modulation of HIV replication by Nef (109,116,120,121,336) . This phenomenon is not species specific as Nef can downregulate CD4 expression in murine cells (11,98,174).

Downregulation of cell surface expression of CD4 is a consequence of its increased internalization induced by Nef (5,242). As mentioned in the first part of the introduction, PMA treatment also leads to an increase in internalization of CD4 by inducing phosphorylation of three serine residues in the cytoplasmic domain of CD4. However, internalization of CD4 induced by Nef was found to be independent of the presence of these serine residues (99). Mutagenesis studies have demonstrated that the presence of two leucine residues at positions 413 and 414 of the cytoplasmic domain of CD4 are required to observe downregulation of CD4 induced by Nef (5). Interestingly, this di-leucine motif was previously described to target cell surface molecules to lysosomal degradation (171). Moreover, Nef was demonstrated to induce degradation of CD4 (242,258,268). The fact that this degradation can be inhibited by inhibitors of lysosomal function (chapter 7) and that CD4 was found to be localized in the endosomes by confocal microscopy, strongly suggest that this degradation of CD4 occurs in such compartments.

Downregulation of mouse CD4 was clearly demonstrated in transgenic mice expressing Nef (33,174,281). Moreover, expression of Nef led to a severely impaired

thymic maturation and altered response of thymocytes and peripheral T cells to mitogenic stimuli. Whether these effects were due only to downregulation of CD4 expression or to other effect of Nef on these T cells (see below) is not determined at this moment.

The role of this downregulation of CD4 and its implication in the effect of Nef on HIV replication is not characterized yet. A report suggested that it prevented viral super infection in the SIV model (25). The fact that this property of Nef is conserved among both laboratory and clinical isolates suggest that it indeed is relevant to the effect of Nef on HIV replication. Models will be proposed in the following chapters.

7.6.3 The effect of Nef on HIV replication

Nef was first identified as a negative regulator of HIV replication, thus its name "Negative factor" (47,178). It was reported that Nef negatively affected transcription from the HIV LTR and was acting as a transcriptional silencer of the HIV LTR (4,178,214). These results suggested that Nef was important in the phenomenon of latency.

Moreover, Nef expression prevents IL-2 production induced by TcR stimulation in cell lines by inhibiting transcription from the IL-2 promoter (182). The expression of Nef was shown to directly inhibit DNA binding activity of NF-κB and AP-1 (212,213). These results further implicate Nef as a negative regulator of HIV replication. Nef can also inhibit activation pathways involved in cell proliferation. Indeed, Nef was shown to inhibit both proliferation and calcium influx in fibroblasts, and IL-2 induced growth in T cells (71). Taken together, these results favored the hypothesis that Nef was involved in latency by inhibiting both transcription from the HIV LTR and activation of T cells.

However, several studies contradict these reports. Indeed, two groups could not observe a negative effect of Nef on either HIV LTR transcription or HIV replication (46,118). Moreover, a positive effect of Nef on HIV replication *in vitro* was observed using Nef sequences isolated from several clinical isolates (70,196,288,337). However, the positive effect of Nef on HIV replication can be harder to observe using T cell lines (70). Moreover, it was clearly demonstrated that the positive effect of Nef on replication is more marked when resting PBLs are infected versus activated PBLs (288,337). The positive effect of Nef thus seems to depend on the activation state of the cell infected. How Nef upregulates HIV replication in vitro is not characterized yet but it seems that the presence of Nef increases the infectivity of the virions produced.

The importance of a functional Nef for HIV infection *in vivo* was clearly demonstrated using SIVmac 239 infection of adult rhesus monkeys as a model (144). Indeed, Nef was required in order to maintain high virus loads and for full pathogenic

potential although *in vitro* infection with the same virus was not altered by the absence of Nef. Moreover, infection with a virus in which a stop codon was inserted in the early in the Nef sequence resulted in reversion of this stop codon to a coding codon. The importance of Nef was further demonstrated using the SCID-hu model where again the presence of Nef was required to observe pathogenesis (137).

Models of the possible mechanism(s) by which Nef exerts its effect on HIV replication will be proposed and discussed in the General Discussion.

8. RATIONALE

Throughout evolution organisms had to discriminate self from non-self to protect their species. The immune system has evolved into a complex ensemble consisting of many lines of defense each specialized for a particular kind of aggression. Pathogens have also evolved so as to manipulate the host defense in their own purpose. The object of this thesis was to understand the role of a cell surface molecule, CD4, in the normal immune response and in a pathogenic situation, namely HIV infection. In addition, the effects of viral proteins on CD4 were characterized. The CD4 molecule is expressed on the surface of T cells that recognize antigen presented by MHC class II molecules. Its association with the tyrosine kinase lck makes it an ideal candidate to generate signals regulating T cell activation since tyrosine kinases, including lck, were demonstrated to be the initiators of T cell activation.

The work presented in this thesis was meant to answer two questions regarding the role of CD4 in T cell activation. The first was to assess specifically the function of the interaction between lck and CD4 in regulating early events of T cell activation. This was accomplished by generating a mutant of CD4 which has lost the ability to associate with lck and studying the effect of the mutation on TcR-induced proliferation of a T cell clone.

The second question we asked was whether CD4 can regulate the initiation of T cell activation independently of its association with lck and of its interaction with MHC class II molecules. This was achieved by substituting the extracellular domain of CD4 with the extracellular domain of a molecule which is not normally found on the surface of T cells (the EGF receptor), and assessing whether there was a loss of function of CD4 in an MHC independent T cell stimulation when this chimeric molecule was expressed.

The fact that the CD4 molecule is the receptor for the HIV has consequences on the regulation of HIV replication *in vivo*. Indeed, several HIV proteins such as gp120 and Nef affect the normal function of CD4. This may result in modulation of the immune response against HIV and other pathogens, thus indirectly regulating the levels of replication of HIV. At a cellular level, the replication of HIV requires activation of the infected T cell. Indeed, cell division and cellular transcription factors only found in activated T cells are required to get productive infection by HIV. These observations suggest that signals generated through CD4 that modulate T cell activation , are also involved in the regulation of HIV replication.

gp120 was shown to directly inhibit the activation of CD4 positive T cells. Although a correlation can be found between activation of lck by the interaction of gp120 with CD4 and the induction of anergy by gp120, no direct proof of the causative effect of

the activation of lck on the induction of anergy has been reported. We have further characterized this mechanism of inhibition of antigenic stimulation using a T cell clone transfected with the above described mutants. As both the HIV LTR and the IL-2 promoter are regulated by common transcription factors, we then tested if gp120 binding to CD4 could also directly modulate HIV replication.

Another HIV protein, Nef, was demonstrated to modulate CD4. Indeed, Nef induces the internalization and degradation of CD4. Since Nef has a positive effect on HIV replication, we were interested in assessing whether the down regulation of CD4 by Nef is implicated in the positive effect of Nef on HIV replication. To determine that, we first characterized the mechanisms by which Nef down regulates CD4 expression and the domains of CD4 which are required for this effect. As lck activity is required for T cell activation, we also examined the fate of the association of CD4 with lck when Nef is present. Indeed, modulation of lck activity by Nef could also be implicated in the positive effect of Nef on HIV replication.

Characterization of the effect of the signals generated through CD4 in modulating both T cell activation and HIV replication will allow a better understanding of the life cycle of HIV. In addition, the identification of the mechanisms by which HIV modulate these signals may be of potential use in designing vaccines and anti-viral therapies.

9. References

1. Abraham, K. M., S. D. Levin, J. D. Marth, K. A. Forbush, and R. M. Perlmutter. 1991. Thymic tumorigenesis induced by overexpression of $p56^{lck}$. Proc. Natl. Acad. Sci. USA. 88:3977-3981.

2. Abraham, N., M. C. Miceli, J. R. Parnes, and A. Veillette. 1991. Enhancement of T-cell responsiveness by the lymphocyte-specific tyrosine protein kinase $p56^{lck}$. Nature 350:62-66.

3. Abraham, N. and A. Veillette. 1990. Activation of $p56^{lck}$ through mutation of a regulatory carboxy-terminal tyrosine residue requires intact sites of autophosphorylation and myristylation. Mol. Cell Biol. 10:5197-5206.

4. Ahmad, N. and S. Venkatesan. 1988. Nef protein of HIV-1 is a transcriptional repressor of HIV-1 LTR . Science 241:1481-1485.

5. Aiken, C., J. Konner, N. R. Landau, M. E. Lenburg, and D. Trono. 1994. Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. Cell 76:853-864.

6. Allan, J. S., J. E. Coligan, T. H. Lee, M. F. McLane, P. J. Kaniki, J. E. Groopman, and M. Essex. 1985. A new HTLV-III/LAV encoded antigen detected by antibodies from AIDS patients. Science 230:810-813.

7. Ameisen, J. C. and A. Capron. 1991. Cell dysfunction and depletion in AIDS: the programmed cell death hypothesis. Immunology Today 12:102-105.

8. Amrein, K. E., N. Flint, B. Panholzer, and P. Burn. 1992. Ras GTPase-activating protein: a substrate and a potential binding protein of the protein-tyrosine kinase $p56^{lck}$. Proc. Natl. Acad. Sci. USA 89:3343-3346.

9. Amrein, K. E., B. Panholzer, N. A. Flint, W. Bannwarth, and P. Burn. 1993. The Src homology 2 domain of the protein-tyrosine kinase $p56^{lck}$ mediates both intermolecular and intramolecular interactions. Proc. Natl. Acad. Sci. USA 90:10285-10289.

10. Anderson, P., M. L. Blue, and S. F. Schlossman. 1988. Comodulation of CD3 and CD4. Evidence for a specific association between CD4 and approximatively 5% of the CD3:T cell receptor complexes on helper T lymphocytes. J. Immunol. 140:1732-1737.

11. Anderson, S., D. C. Shugars, R. Swanstrom, and J. V. Garcia. 1993. Nef from primary isolates of human immunodeficiency virus type 1 suppresses surface CD4 expression in human and mouse T cells. J. Virol. 67:4923-4931.

12. Anderson, S. J., S. D. Levin, and R. M. Perlmutter. 1993. Protein tyrosine kinase $p56^{lck}$ controls allelic exclusion of receptor β-chain genes. Nature 365:552-554.

13. Appleby, M. W., J. Gross, M. P. Cooke, S. D. Levin, X. Qian, and R. M. Perlmutter. 1992. Defective T cell receptor signaling in mice lacking the thymic isoform of $p59^{fyn}$. Cell 70:751-763.

14. Arpaia, E., M. Shahar, H. Dadi, A. Cohen, and C. M. Roifman. 1994. Defective T cell receptor signaling and CD8+ thymic selection in humans lacking ZAP-70 kinase. Cell 76:947-958.

15. Arthos, J., K. C. Deen, M. A. Chaikin, J. A. Fornwald, G. Sathe, Q. J. Sattentau, P. R. Clapham, R. A. Weiss, J. S. McDougal, C. Pietropaolo, R. Axel, A. Truneh, P. J. Maddon, and R. W. Sweet. 1989. Identification of the residues in human CD4 critical for the binding of HIV. Cell 57:469-481.

16. Ashkenazi, A., L. G. Presta, S. A. Marsters, T. R. Camerato, K. A. Rosenthal, B. M. Fendly, and D. J. Capon. 1990. Mapping the CD4 binding site for human immunodeficiency virus by alanine-scanning mutagenesis. Proc. Natl. Acad. Sci. USA 87:7150-7154.

17. Autero, M., J. Saharinen, T. Pessa-Morikawa, M. Soula-Rothhut, C. Oetken, M. Gassman, M. Bergman, K. Alitalo, P. Burn, C. G. Gahmberg, and T. Mustelin. 1994. Tyrosine phosphorylation of CD45 phosphotyrosine phosphatase by $p50^{csk}$ creates a binding site for $p56^{lck}$ tyrosine kinase and activates the phosphatase. Mol. Cell. Biol. 14:1308-1321.

18. Azuma, M., M. Cayabyab, D. Buck, J. H. Phillips, and L. L. Lanier. 1992. CD28 interaction with B7 costimulates primary allogeneic proliferative responses and cytotoxicity mediated by small, resting T lymphocytes. Journal of Experimental Medecine 175:353-360.

19. Baldari, C. T., A. Heguy, and J. L. Telford. 1993. Calcium-dependent cyclosporin A sensitive activation of the Il-2 promoter by $p56^{lck}$. J. Biol. Chem. 268:8406-8409.

20. Banda, N. K., J. Bernier, D. K. Kurahara, R. Kurrle, W. Haigwood, R. P. Sekaly, and T. Finkel. 1992. Crosslinking CD4 by human immunodeficiency virus gp120 primes T cells for activation-induced apoptosis. J. Exp. Med. 176:1099-1106.

21. Bandres, J. C. and L. Ratner. 1994. Human immunodeficiency virus type 1 Nef protein down-regulates transcription factors NF-κB and AP-1 in human T cells in vitro after T-cell receptor stimulation. J. Virol. 68:3243-3249.

22. Bauer, A., D. J. McConkey, F. D. Howard, L. K. Clayton, D. Novick, S. Koyasu, and E. L. Reinherz. 1991. Differential signal transduction via T-cell receptor CD3 ζ/ζ, CD3 ζ-η, and CD3 η/η isoforms. Proc. Natl. Acad. Sci. USA. 88:3842-3846.

23. Beaufils, P., D. Choquet, R. Z. Mamoun, and B. Malissen. 1993. The (YXXL/I)2 signalling motif found in the cytoplasmic segments of the bovine leukaemia virus envelope protein and Epstein-Barr virus latent membrane protein 2A can elicit early and late lymphocyte activation events. EMBO J. 12:5105-5112.

24. Bedinger, P., A. Moriarty, R. C. von Borstel II, N. J. Donovan, K. S. Steimer, and D. Littman,R. 1988. Internalization of the human immunodeficiency virus does not require the cytoplasmic domain of CD4. Nature 334:162-164.

25. Benson, R. E., A. Sanfridson, J. S. Ottinger, C. Doyle, and B. R. Cullen. 1993. Downregulation of cell-surface CD4 expression by simian immunodeficiency virus Nef prevents viral super infection. J. Exp. Med. 177:1561-1566.

26. Benveniste, P., Y. Takahama, D. L. Wiest, T. Nakayama, S. O. Sharrow, and A. Singer. 1994. Engagement of the external domains of CD45 tyrosine phosphatase can regulate the differentiation of immature CD4+CD8+ thymocytes into mature T cells. Proc. Natl. Acad. Sci. USA. 91:6933-6937.

27. Bergman, M., T. Mustelin, C. Oetken, J. Partanen, N. A. Flint, K. E. Amrein, M. Autero, P. Burn, and K. Alitalo. 1992. The human $p50^{csk}$ tyrosine kinase phosphorylates $p56^{lck}$ at Tyr-505 and down regulates its catalytic activity. EMBO J. 11:2919-2924.

28. Blue, M.-L., D. A. Hafler, K. A. Craig, H. Levine, and S. F. Schlossman. 1987. Phosphorylation of CD4 and CD8 molecules following T cell triggering. Journal of Immunology 139:3949-3954.

29. Blum, M. D., G. T. Wong, K. M. Higgins, M. J. Sunshine, and E. Lacy. 1993. Reconstitution of subclass specific expression of CD4 in thymocytes and peripheral T cells of transgenic mice: identification of a human CD4 enhancer. J. Exp. Med. 177:1343-1358.

30. Bour, S., F. Boulerice, and M. A. Wainberg. 1991. Inhibition of gp160 and CD4 maturation in U937 cells after both defective and productive infections by HIV-1. J. Virol. 65:6387-6396.

31. Boussiotis, V. A., G. J. Freeman, G. Gray, J. Gribben, and L. Nadler. 1993. B7 but not intercellular adhesion molecule-1 costimulation prevents the induction of human alloantigen-specific tolerance. Journal of Experimental Medecine 178:1753-1763.

32. Bowman, M. R., K. D. MacFerrin, S. L. Schreiber, and S. J. Burakoff. 1990. Identification and structural analysis of residues in the V1 region of CD4 involved in interaction with human immunodeficiency virus envelope glycoprotein gp120 and class II major histocompatibility complex molecules. Proc. Natl. Acad. Sci. USA 87:9052-9056.

33. Brady, H. J., D. J. Pennington, C. G. Miles, and E. A. Dzierzak. 1993. CD4 cell surface downregulation in HIV-1 Nef transgenic mice is a consequence of intracellular sequestration. EMBO J. 12:4923-4932.

34. Brady, R. L., E. J. Dodson, G. G. Dodson, and G. Lange. 1993. Crystal structure of domains 3 and 4 of rat CD4 : Relationship to the NH2-Terminal domains. Science 260:979-983.

35. Brown, J. H., T. S. Jardetzky, J. C. Gorga, L. J. Stern, R. G. Urban, J. L. Strominger, and D. C. Wiley. 1993. Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. Nature 364:33-39.

36. Burgess, E. K., A. D. Odysseos, C. Zalvan, B. J. Drucker, P. Anderson, S. E. Schlossman, and C. E. Rudd. 1991. Biochemical identification of a direct physical interaction between the CD4:p56^{lck} and Ti(TcR)/CD3 complexes. Eur. J. Immunol. 21:1663-1668.

37. Burkhard, A. L., B. Stealy, R. B. Rowley, S. Mahajan, M. Prendergast, J. Fargnoli, and J. B. Bolen. 1994. Temporal regulation of non-transmembrane protein tyrosine kinase enzyme activity following T cell antigen receptor engagement. J. Biol. Chem. 269:23642-23647.

38. Burns, C. M., K. Sakaguchi, E. Appella, and J. D. Ashwell. 1994. CD45 regulation of tyrosine phosphorylation and enzyme activity of src family kinases. Journal of Biological Chemistry. 269:13594-13600.

39. Callebaut, C., B. Krust, E. Jacotot, and A. G. Hovanessian. 1993. T cell activation antigen, CD26, as a cofactor for entry of HIV in CD4+ cells. Science 262:2045-2050.

40. Caron, L., N. Abraham, T. Pawson, and A. Veillette. 1992. Structural requirements for enhancement of T-cell responsiveness by the lymphocyte-specific tyrosine protein kinase p56^{lck}. Mol. Cell. Biol. 12:2720-2729.

41. Cefai, D., M. Ferrer, N. Serpente, T. Idziorek, A. Dautry-Varsat, P. Debre, and G. Bismuth. 1992. Internalization of HIV glycoprotein gp120 is associated with down modulation of membrane CD4 and p56^{lck} together with impairment of T cell activation. J. Immunol. 149:285

42. Chan, A. C., B. A. Irving, J. D. Fraser, and Weiss. 1991. The zeta chain is associated with a tyrosine kinase and upon T-cell antigen receptor stimulation associates with ZAP-70, a 70-kDa tyrosine phosphoprotein. Proc. Natl. Acad. Sci. USA. 88:9166-9170.

43. Chan, A. C., M. Iwashima, C. W. Turck, and A. Weiss. 1992. ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR ζ chain. Cell 71:649-662.

44. Chan, A. C., T. A. Kadlecek, M. E. Elder, A. H. Filipovich, W. L. Kuo, M. Iwashima, T. G. Parslow, and A. Weiss. 1994. ZAP-70 deficiency in an autosomal recessive form of severe combined immunodeficiency. Science 264:1599-1601.

45. Chan, A. C., N. S. van Oers, A. Tran, L. Turka, C. L. Law, J. C. Ryan, E. A. Clark, and A. Weiss. 1994. Differential expression of ZAP-70 and Syk protein tyrosine kinases, and the role of this family of protein tyrosine kinases in TCR signaling. J. Immunol. 152:4758-4766.

46. Chang, J. C., L. Zhang, T. L. Edgerton, and A. M. Kaplan. 1990. Heterogeneity in direct cytotoxic function of L3T4 T cells. TH1 clones express higher cytotoxic activity to antigen-presenting cells than TH2 clones. Journal of Immunology 145:409-416.

47. Cheng-Mayer, C., P. Iannello, K. Shaw, P. A. Luciw, and J. A. Levy. 1989. Differential effects of nef on HIV replication: implications for viral pathogenesis in the host. Science 246:1629-1632.

48. Chimurle, N., V. S. Kalyanaraman, N. Oyaizu, H. B. Slade, and S. Pahwa. 1990. Inhibition of functional properties of tetanus antigen-specific T cell clones by envelope glycoprotein gp120 of human immunodeficiency virus. Blood 75:152

49. Chow, L. M. L., C. Jarvis, Q. Hu, S. H. Nye, F. G. Gervais, A. Veillette, and L. A. Matis. 1994. ntk: a csk related protein tyrosine kinase expressed in brain and T lymphocytes. Proc. Natl. Acad. Sci. USA 91:4975-4979.

50. Chow, L. M. M., M. Fournel, D. Davidson, and A. Veillette. 1993. Negative regulation of T-cell receptor signalling by tyrosine protein kinase p50^{csk}. Nature 365:156-160.

51. Chu, K. and D. R. Littman. 1994. Requirement for kinase activity of CD4-associated p56lck in antibody-triggered T cell signal transduction. Journal of Biological Chemistry. 269:24095-24101.

52. Clayton, L. K., R. E. Hussey, R. Steinbrich, H. Ramachandran, Y. Husain, and E. L. Reinherz. 1988. Substitution of murine for human CD4 residues identifies amino acids critical for HIV-gp120 binding. Nature 335:363-366.

53. Clayton, L. K., M. Sieh, D. A. Pious, and E. L. Reinherz. 1989. Identification of human CD4 residues affecting class II MHC versus HIV-1 gp120 binding. Nature 339:548-551.

54. Collins, T. L., S. Uniyal, J. Shin, J. L. Strominger, R. S. Mittler, and S. J. Burakoff. 1992. $p56^{lck}$ association with CD4 is required for the interaction between CD4 and the TcR/CD3 complex and for optimal antigen stimulation. J. Immunol 148:2159-2162.

55. Cooke, M. P., K. M. Abraham, K. A. Forbush, and R. M. Perlmutter. 1991. Regulation of T cell receptor signaling by a src family protein-tyrosine kinase ($p59^{fyn}$). Cell 65:281-291.

56. Cooke, M. P. and R. M. Perlmutter. 1989. Expression of a novel form of the fyn proto-oncogene in hematopoietic cells. New Biol. 1:66-74.

57. Cooper, J. A. and B. Howell. 1993. The when and how of Src Regulation. Cell 73:1051-1054.

58. Cosgrove, D., D. Gray, A. Dierich, J. Kaufman, M. Lemeur, C. Benoist, and D. Mathis. 1991. Mice lacking MHC class II molecules. Cell 66:1051-1066.

59. Couture, C., G. Baier, C. Oetken, S. Williams, D. Telford, A. Marie-Cardine, G. Baier-Bitterlich, S. Fischer, P. Burn, and A. Altman. 1994. Activation of $p56^{lck}$ by $p72^{syk}$ through physical association and N-terminal tyrosine phosphorylation. Mol. Cell Biol. 14:5249-5258.

60. Crise, B., L. Buonocore, and J. K. Rose. 1990. CD4 is retained in the endoplasmic reticulum by the human immunodeficiency virus type 1 glycoprotein precursor. J. Virol. 64:5585-5593.

61. Cullen, B. R. 1991. Regulation of human immunodeficiency virus replication. Annu. Rev. Microbiol. 45:219-250.

62. Dalgleish, A. G., P. C. L. Beverley, P. R. Clapham, D. H. Crawford, M. F. Greaves, and R. A. Weiss. 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature 312:763-766.

63. Damle, N. K., K. Klussman, P. S. Linsley, and A. Aruffo. 1992. Differential costimulatory effects of adhesion molecules B7, ICAM-1, LFA-3, and VCAM-1 on resting and antigen-primed CD4+ T lymphocytes. Journal of Immunology 148:1985-1992.

64. Danielian, S., A. Alcover, L. Polissard, M. Stefanescu, O. Acuto, S. Fischer, and R. Fagard. 1992. Both T cell receptor (TcR)-CD3 complex and CD2 increase the tyrosine kinase activity of $p56^{lck}$. CD2 can mediate TcR-CD3-independent and CD45-dependent activation of $p56^{lck}$. European Journal of Immunology 22:2915-2921.

65. Danska, J. A., A. M. Livingston, V. Paragas, I. Toshiki, and C. Garrison Fathman. 1990. The Presumptive CDR3 Regions of Both T Cell Receptor α and β Chains Determine T Cell Specificity for Myoglobulin Peptides. Journal of Experimental Medecine 172:27-33.

66. Dasgupta, J. D., C. Granja, B. Druker, L. L. Lin, E. J. Yunis, and V. Relias. 1992. Phospholipase C-γ1 association with CD3 structure in T cells. Journal of Experimental Medicine 175:285-288.

67. Davidson, D., L. M. L. Chow, M. Fournel, and A. Veilette. 1992. Differential regulation of T cell antigen responsiveness by isoforms of the src-related tyrosine protein kinase. J. Exp. Med. 175:1483-1492.

68. Davis, S. J., R. L. Brady, A. N. Barclay, K. Harlos, G. G. Dodson, and A. F. Williams. 1990. Crytallization of a soluble form of the rat T-cell surface glycoprotein CD4 complexed with Fab from the W3/25 monoclonal antibody. J. Mol. Biol. 213:7-10.

69. Davodeau, F., M. A. Peyrat, F. Romagne, A. Necker, M. M. Hallet, H. Vie, and M. Bonneville. 1995. Dual T cell receptor β chain expression on human T lymphocytes. Journal of Experimental Medicine 181:1391-1398.

70. de Ronde, A., B. Klaver, W. Keulen, L. Smit, and J. Goudsmit. 1992. Natural HIV-1 NEF accelerates virus replication in primary human lymphocytes. Virology 188:391-395.

71. De, S. K. and J. W. Marsh. 1994. HIV-1 Nef inhibits a common activation pathway in NIH-3T3 cells. J. Biol. Chem. 269:6656-6660.

72. Desai, D. M., J. Sap, J. Schlessinger, and A. Weiss. 1993. Ligand-mediated negative regulation of a chimeric transmembrane receptor tyrosine phosphatase. Cell 73:541-554.

73. Diamond, D. C., R. Finberg, S. Chaudhuri, B. P. Sleckman, and S. J. Burakoff. 1990. Human immunodeficiency virus infection is efficiently mediated by a glycolipid-anchored form of CD4. Proc. Natl. Acad. Sci. USA 87:5001-5005.

74. Dianzani, U., U. Moglia, F. Malavasi, M. Bragardo, A. Pileri, C. A. Janeway, and K. Bottomly. 1992. Isoform-specific associations of CD45 with accessory molecules in human T lymphocytes. Eur. J. Immunol. 22:365-371.

75. Dianzani, U., A. Shaw, B. K. al-Ramadi, R. T. Kubo, and C. A. Janeway,Jr. 1992. Physical association of CD4 with the T cell receptor. J. Immunol. 148:678-688.

76. Diez-Orejas, R., S. Ballester, M. J. Feito, G. Ojeda, G. Criado, M. Ronda, P. Portoles, and J. M. Rojo. 1994. Genetic and immunochemical evidence for CD4-dependent association of $p56^{lck}$ with the T cell receptor:regulation of TcR induced activation. EMBO J. 13:90-99.

77. Downward, J., J. D. Graves, P. H. Warne, S. Rayter, and D. A. Cantrell. 1990. Stimulation of p21ras upon T-cell activation . Nature 346:719-723.

78. Doyle, C. and J. L. Strominger. 1987. Interaction between CD4 and class II MHC molecules mediates cell adhesion. Nature 330:256-259.

79. Duplay, P., M. Thome, F. Herve, and O. Acuto. 1994. p56^{lck} interacts via its src homology 2 domain with the ZAP-70 kinase. Journal of Experimental Medicine 179:1163-1172.

80. Eck, M. J., S. K. Atwell, S. E. Shoelson, and S. C. Harrison. 1994. Structure of the regulatory domains of the Src-family tyrosine kinase Lck. Nature 368:764-769.

81. Elder, M. E., D. Lin, J. Clever, A. C. Chan, T. J. Hope, A. Weiss, and T. G. Parslow. 1994. Human severe combined immunodeficiency due to a defect in ZAP-70, a T cell tyrosine kinase. Science 264:1596-1599.

82. Ellis, C., X. Q. Liu, D. Anderson, N. Abraham, A. Veillette, and T. Pawson. 1991. Tyrosine phosphorylation of GAP and GAP-associated proteins in lymphoid and fibroblast cells expressing lck. Oncogene 6:895-901.

83. Embretson, J., M. Zupanic, J. L. Ribas, A. Burke, P. Racz, K. Tenner-Racz, and A. T. Haase. 1993. Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. Nature 362:359-361.

84. Engel, I. and S. M. Hendrick. 1988. Site-Directed Mutations in the VDJ Junctionnal Region of a T cell Receptor β Chain Cause Changes in Antigenic Peptide Recognition. Cell 54:473-484.

85. Eshhar, Z., T. Waks, G. Gross, and D. G. Schindler. 1993. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the γ or ζ subunits of the immunoglobulin and T cell receptors. Proc. Natl. Acad. Sci. USA 90:720-724.

86. Ettehadieh, E., J. S. Sanghera, S. L. Pelech, D. Hess-Bienz, J. Watts, N. Shastri, and R. Aebersold. 1992. Tyrosyl phosphorylation and activation of MAP kinases by p56^{lck}. Science 255:853-855.

87. Evavold, B. D. and P. M. Allen. 1991. Separation of IL-4 production from Th cell proliferation by an altered T cell receptor ligand. Science 252:1308-1310.

88. Evavold, B. D., J. Sloan-Lancaster, B. L. Hsu, and P. M. Allen. 1993. Separation of T helper 1 clone cytolysis from proliferation and lymphokine production using analog peptides. J. Immunol. 150:3131-3140.

89. Fauci, A. S. 1988. The human immunodeficiency virus : infectivity and mechanisms of pathogenesis. Science 239:617-622.

90. Feinberg, M. B., R. F. Jarrett, A. Aldovini, R. C. Gallo, and F. Wong-Staal. 1986. HTLV-III expression and production involve complex regulation at the levels of splicing and translation of viral RNA. Cell 46:807-817.

91. Fields, A. P., D. P. Bednarik, A. Hess, and W. S. May. 1988. Human immunodeficiency virus induces phosphorylation of its cell surface receptor. Nature 333:278-280.

92. Fleury, S., D. Lamarre, S. Meloche, S.-E. Ryu, C. Cantin, W. A. Hendrickson, and R.-P. Sekaly. 1991. Mutational analysis of the interaction between CD4 and class II MHC: class II antigens contact CD4 on a surface opposite to the gp120-binding site. Cell 66:1037-1049.

93. Franchini, G., G. M. Robert, J. Ghrayeb, N. T. Chang, and F. Wong-Staal. 1986. Cytoplasmic localization of the 3`orf protein in cultured T cells. Virology 155:593-599.

94. Frank, S. J., B. B. Niklinska, D. G. Orloff, M. Mercep, D. Ashwell, and R. D. Klausner. 1990. Structural mutations of the T cell receptor ζ chain and its role in T cell activation. Science 249:174-177.

95. Freeman, G. J., J. Gribben, V. A. Boussiotis, J. W. NG, V. A. J. Restivo, D. B. Lombard, G. Gray, and L. M. Nadler. 1993. Cloning of B7-2: a CTLA-4 counter-receptor that costimulates human T cell proliferation. Science 262:909-911.

96. Fung-Leung, W. P., M. W. Schilham, A. Rahemtulla, T. M. Kundig, M. Vollenweider, J. Potter, W. van Ewijk, and T. W. Mak. 1991. CD8 is needed for development of cytotoxic T cells but not helper T cells. Cell 65:443-9.

97. Gajewski, T. F., D. Qian, P. Fields, and F. W. Fitch. 1994. Anergic T lymphocytes clones have altered inositol phosphate, calcium, and tyrosine signaling pathways. Proc. Natl. Acad. Sci. USA 91:38-42.

98. Garcia, J. V., J. Alfano, and A. D. Miller. 1993. The negative effect of human immunodeficiency virus type 1 Nef on cell surface CD4 expression is not species specific and requires the cytoplasmic domain of CD4. J. Virol. 67:1511-1516.

99. Garcia, J. V. and A. D. Miller. 1991. Serine phosphorylation-independent downregulation of cell-surface CD4 by nef. Nature 350:508-511.

100. Gay, D., S. Buss, J. Pasternak, J. Kappler, and P. Marrack. 1988. The T cell accessory molecule CD4 recognizes a monomorphic determinant on isolated Ia. Proc. Natl. Acad. Sci. USA 85:5629

101. Geleziunas, R., S. Bour, F. Boulerice, J. Hiscott, and M. A. Wainberg. 1991. Diminution of CD4 surface protein but not CD4 mRNA levels in monocytic cells infected by HIV-1. AIDS 5:29-33.

102. Geleziunas, R., S. Bour, and M. A. Wainberg. 1994. Cell surface down-modulation of CD4 after infection by HIV-1. FASEB J. 8:593-600.

103. Germain, R. N. 1994. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocytes activation. Cell 76:287-299

104. Gervais, F. G., L. M. Chow, J. M. Lee, P. E. Branton, and A. Veillette. 1993. The SH2 domain is required for stable phosphorylation of $p56^{lck}$ at tyrosine 505, the negative regulatory site. Mol. Cell Biol. 13:7112-7121.

105. Glaichenhaus, N., N. Shastri, D. R. Littman, and J. M. Turner. 1991. Requirement for association of $p56^{lck}$ with CD4 in antigen-specific signal transduction in T cells. Cell 64:511-520.

106. Goldman, F., W. A. Jensen, G. L. Johnson, L. Heasley, and J. C. Cambier. 1994. gp120 ligation of CD4 induces $p56^{lck}$ activation and TcR desensitization independent of TcR tyrosine phosphorylation. J. Immunol 153:2905-2917.

107. Goldman, S. J., S. Uniyal, L. M. Ferguson, D. E. Golan, S. J. Burakoff, and P. A. Kiener. 1992. Differential activation of phosphotyrosine protein phosphatase activity in a murine T cell hybridoma by monoclonal antibodies to CD45. Journal of Biological Chemistry. 267:6197-6204.

108. Greenstein, J. L., J. Kappler, P. Marrack, and S. J. Burakoff. 1984. The role of L3T4 in recognition of Ia by a cytotoxic, H-2D^{d}-specific T cell hybridoma. J. Exp. Med. 159:1213-1224.

109. Greenway, A. L., D. A. McPhee, E. Grgacic, D. Hewish, A. Lucantoni, I. Macreadie, and A. Azad. 1994. Nef 27, but not the Nef 25 isoform of human immunodeficiency virus-type 1 pNL4.3 down-regulates surface CD4 and IL-2R expression in peripheral blood mononuclear cells and transformed T cells. Virology 198:245-256.

110. Gross, J. A., M. W. Appleby, S. Chien, S. Nada, S. H. Bartelmez, M. Okada, S. Aizawa, and R. M. Perlmutter. 1995. Control of lymphopoesis by p50^{csk}, a regulatory protein tyrosine kinase. J. Exp. Med. 181:463-473.

111. Groux, H., G. Torpier, D. Monte, Y. Mouton, A. Capron, and J. C. Ameisen. 1992. Activation-induced death by apoptosis in CD4+ T cells from human immunodeficiency virus-infected asymptomatic individuals. Journal of Experimental Medicine 175:331-40.

112. Grusby, M. J., R. S. Johnson, V. E. Papaionnou, and L. H. Glimcher. 1991. Depletion of CD4+ T cells in major histocompatibility complex class II-deficient mice. Science 253:1417-1420.

113. Gulbins, E., K. M. Coggeshall, G. Baier, S. Katzav, P. Burn, and A. Altman. 1993. Tyrosine kinase stimulated guanine nucleotide exchange activity of vav in T cell activation. Science 260:822-825.

114. Gupta, S. and B. Safai. 1983. Deficient autologous mixed lymphocyte reaction in Kaposi's sarcoma associated with deficiency of leu3 positive responder T cells. J. Clin. Invest. 71:296

115. Guy, B., M. P. Kieny, Y. Riviere, C. LePeuch, K. Dott, M. Girard, L. Montagnier, and J. P. Lecocq. 1987. HIV F/3` orf encodes a phosphorylated GTP-binding protein resembling an oncogene product. Nature 330:266-269.

116. Guy, B., Y. Riviere, K. Dott, A. Regnault, and M. P. Kieny. 1990. Mutational analysis of the HIV nef protein. Virology 176:413-425.

117. Hall, C. G., J. Sancho, and C. Terhorst. 1993. Reconstitution of T cell receptor ζ-mediated calcium mobilization in non lymphoid cells. Science 261:915-917.

118. Hammes, S. R., E. P. Dixon, M. H. Malim, B. R. Cullen, and W. C. Greene. 1989. Nef protein of human immunodeficiency virus type 1: evidence against its role as a transcriptional inhibitor. Proc. Natl. Acad. Sci. U. S. A. 86:9549-9553.

119. Harding, F. A., J. G. McArthur, J. A. Gross, D. H. Raulet, and J. P. Allison. 1992. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. Nature 356:607-609.

120. Harris, M. and K. Coates. 1993. Identification of cellular proteins that bind to the human immunodeficiency virus type 1 nef gene product in vitro: a role for myristylation. J. Gen. Virol. 74:1581-1589.

121. Harris, M. P. and J. C. Neil. 1994. Myristoylation-dependent binding of HIV-1 Nef to CD4. J. Mol. Biol. 241:136-142.

122. Hatakeyama, M., T. Kono, N. Kobayashi, A. Kawahara, S. D. Levin, R. M. Perlmutter, and T. Taniguchi. 1991. Interaction of the IL-2R receptor with the src-family kinase $p56^{lck}$: identification of novel intermolecular association. Science 252:1523-1528.

123. Hermans, M. H. A. and B. Malissen. 1993. The cytoplasmic tail of the T cell receptor ζ chain is dispensible for antigen mediated T cell activation. Eur. J. Immunol. 23:2257-2262.

124. Hivroz, C., F. Mazerolles, M. Soula, R. Fagard, S. Gratton, S. Meloche, R. P. Sekaly, and A. Fisher. 1993. Human immunodeficiency virus gp120 derived peptides activate protein tyrosine kinase $p56^{lck}$ in human CD4 lymphocytes. Eur. J. Immunol. 23:600-607.

125. Ho, D. D., A. U. Neumann, A. S. Perelson, W. Chen, J. M. Leonard, and M. Markowitz. 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature 373:123-126.

126. Horak, I. D., R. E. Gress, P. J. Lucas, E. M. Horak, T. A. Waldmann, and J. B. Bolen. 1991. T-lymphocyte interleukin 2-dependent tyrosine protein kinase signal transduction involves the activation of $p56^{lck}$. Proc. Natl. Acad. Sci. U. S. A. 88:1996-2000.

127. Hovis, R. R., J. A. Donovan, M. A. Musci, D. G. Motto, F. D. Goldman, S. E. Ross, and G. A. Koretzky. 1993. Rescue of signaling by a chimeric protein containing the cytoplasmic domain of CD45. Science 260:544-546.

128. Hoxie, J. A., J. D. Alpers, J. L. Rackowski, K. Huebner, B. S. Haggarty, A. J. Cedarbaum, and J. C. Reed. 1986. Alterations in T4 (CD4) protein and mRNA synthesis in cells infected with HIV. Science 234:1123-1127.

129. Hurley, T. R., R. Hyman, and B. M. Sefton. 1993. Differential effects of expression of the CD45 tyrosine protein phosphatase on the tyrosine phosphorylation of the lck, fyn, and c-src tyrosine protein kinases. Mol. Cell Biol. 13:1651-1656.

130. Hurley, T. R., K. Luo, and M. Sefton. 1989. Activators of protein kinase C induce dissociation of CD4, but not CD8, from $p56^{lck}$. Science 245:407-409.

131. Ingold, A. L., C. Landel, C. Knall, G. A. Evans, and T. A. Potter. 1991. Co-engagement of CD8 with the T cell receptor is required for negative selection. Nature 352:721-723.

132. Irving, B. A., A. C. Chan, and Weiss. 1993. Functional characterization of a signal transducing motif present in the T cell antigen receptor ζ chain. J. Exp. Med. 177:1093-1103.

133. Irving, B. A. and A. Weiss. 1991. The cytoplasmic domain of the T cell receptor ζ chain is sufficient to couple to receptor-associated signal transduction pathways. Cell 64:891-901.

134. Iwashima, M., B. A. Irving, N. S. van Oers, A. C. Chan, and A. Weiss. 1994. Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. Science 263:1136-1139.

135. Jabado, N., F. LeDeist, A. Fisher, and C. Hivroz. 1994. Interaction of HIV gp120 and anti-CD4 antibodies with the CD4 molecule on Human CD4+ T cells inhibits the binding activity of NF-AT, NF-κB, and AP-1, three nuclear factors regulating IL-2 gene enhancer activity. Eur. J. Immunol. 24:2646-2652.

136. Jabbar, M. A. and D. P. Nayak. 1990. Intracellular interaction of human immunodeficiency virus type 1 (ARV-2) envelope glycoprotein gp160 with CD4 blocks the movement and maturation of CD4 to the plasma membrane. J. Virol. 64:6297-6304.

137. Jamieson, B. D., G. M. Aldrovandi, V. Planelles, J. B. Jowett, L. Gao, L. M. Bloch, I. S. Chen, and J. A. Zack. 1994. Requirement of human immunodeficiency virus type 1 nef for in vivo replication and pathogenicity. J. Virol. 68:3478-3485.

138. Jensen, J. P., C. Cenciarelli, D. Hou, B. L. Rellahan, M. Dean, and A. M. Weissman. 1993. T cell antigen receptor-η subunit: low levels of expression and limited cross-species conservation. J. Immunol. 150:122-130.

139. Jorgensen, J. L., U. Esser, B. Fazekas de St.Groth, P. A. Reay, and M. M. Davis. 1992. Mapping T-cell receptor-peptide contacts by variant peptide immunization of single-chain transgenics. Nature 355:224-230.

140. June, C. H., M. C. Fletcher, J. A. Ledbetter, and L. E. Samelson. 1990. Increases in tyrosine phosphorylation are detectable before phospholipase C activation after T cell receptor stimulation. J. Immunol. 144:1591-1599.

141. June, C. H., M. C. Fletcher, J. A. Ledbetter G. L. Schieven, J. N. Siegel, A. F. Phillips, and L. E. Samelson. 1990. Inhibition of tyrosine phosphorylation prevents T cell receptor mediated signal transduction. Proc. Natl. Acad. Sci. USA 87:7722-7726.

142. Juszczak, R., H. Turchin, A. Truneh, J. Culp, and S. Kassis. 1991. Effects of human immunodeficiency virus gp120 glycoprotein on the association of the protein tyrosine kinase $p56^{lck}$ with CD4 in human T lymphocytes. J. Biol. Chem. 266:1

143. Kamitz, L., S. L. Sutor, T. Torigoe, J. C. Reed, M. P. Bell, D. J. McKean, P. J. Leibson, and R. T. Abraham. 1992. Effects of $p56^{lck}$ deficiency on the growth and cytolytic effector function of an interleukin-2-dependent cytotoxic T-cell line. Mol. Cell. Biol. 12:4521-4530.

144. Kestler, H. W., 3d, D. J. Ringler, K. Mori, D. L. Panicali, P. K. Sehgal, M. D. Daniel, and R. C. Desrosiers. 1991. Importance of the *nef* gene for maintenance of high virus loads and for development of AIDS. Cell 65:651-662.

145. Kinch, M. S., A. Sanfridson, and C. Doyle. 1994. The protein tyrosine kinase $p56^{lck}$ regulates cell adhesion mediated by CD4 and major histocompatibility complex class II proteins. J. Exp. Med. 180:1729-1739.

146. Kishihara, K., J. Penninger, V. A. Wallace, T. M. Kundig, K. Kawai, A. Wakeman, E. Timms, K. Pfeffer, P. S. Ohashi, M. L. Thomas, C. Furlonger, C. J. Paige, and T. M. Mak. 1993. Normal B lymphocyte development but impaired T cell maturation in CD45-exon6 protein tyrosine phosphatase-deficient mice. Cell 74:143-156.

147. Klages, S., D. Adam, K. Class, J. Fargnoli, J. B. Bolen, and R. C. Penhallow. 1994. ctk: a protein tyrosine kinase related to csk that defines an enzyme family. Proc. Natl. Acad. Sci. USA 91:2597-2601.

148. Klatzmann, D., E. Champagne, S. Chamaret, J. Gruest, D. Guetard, T. Hercend, J. C. Gluckman, and L. Montagnier. 1984. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature 312:767-768.

149. Kolanus, W., C. Romeo, and B. Seed. 1993. T cell activation by clustered tyrosine kinases. Cell 74:171-183.

150. Koller, B. H., P. Marrack, J. W. Kappler, and O. Smithies. 1990. Normal Development of mice deficient in β2M, MHC class I proteins, and CD8+ T cells. Science 248:1227-1248.

151. Koretzky, G. A., J. Picus, M. L. Thomas, and A. Weiss. 1990. Tyrosine phosphatase CD45 is essential for coupling T-cell antigen receptor to the phosphatidyl inositol pathway. Nature 346:66-68.

152. Kornfeld, H., W. W. Cruikshank, S. W. Pyle, J. S. Berman, and D. M. Center. 1988. Lymphocyte activation by HIV-1 envelope glycoprotein. Nature 335:445-447.

153. Kupfer, A. and S. Singer,J. 1989. The specific interaction of helper T cells and antigen presenting B cells: membrane and cytoskeletal reorganization in the bound T cell as a function of antigen dose. J. Exp. Med. 170:1697-1713.

154. Kupfer, A. and S. J. Singer. 1988. Molecular dynamics in the membrane of helper T-Cells. Proc. Natl. Acad. Sci. USA 85:8216

155. Kupfer, A., S. J. Singer, C. J. Janeway, and S. L. Swain. 1987. Coclustering of CD4 (L3T4) molecule with the T-cell receptor is induced by specific direct interaction of helper T cells and antigen-presenting cells. Proc. Natl. Acad. Sci. USA. 84:5888-5892.

156. Kupfer, A., S. L. Swain, C. A. Janeway, and S. Singer,J. 1986. The specific direct interaction of helper T cells and antigen presenting B cells. Proc. Natl. Acad. Sci. USA 83:6080-6083.

157. Kupfer, A., S. L. Swain, and S. Singer,J. 1987. The specific direct interaction of helper T cells and antigen presenting B cells: reorientation of the microtubule organizing center and reorganization of the membrane-associated cytoskeleton inside the bound helper T cells. J. Exp. Med. 165:1565-1580.

158. Kwong, P. D., S. E. Ryu, W. A. Hendrickson, R. Axel, R. M. Sweet, G. Folena-Wasserman, P. Hensley, and R. W. Sweet. 1990. Molecular characteristics of recombinant human CD4 as deduced from polymorphic crystals. Proc. Natl. Acad. Sci. USA 87:6423-6427.

159. Lamarre, D., A. Ashkenazi, S. Fleury, D. H. Smith, R. P. Sekaly, and D. J. Capon. 1989. The MHC-binding and gp120-binding functions of CD4 are separable. Science 245:743-746.

160. Lamarre, D., D. J. Capon, D. R. Karp, T. Gregory, E. Long, and R. P. Sekaly. 1989. Class II MHC molecules and the HIV gp120 envelope protein interact with functionally distinct regions of the CD4 molecule. EMBO J. 8:3271-3277.

161. Landau, N. R., M. Warton, and D. R. Littman. 1988. The envelope glycoprotein of the human immunodeficiency virus binds to the immunoglobulin-like domain of CD4. Nature 334:159-162.

162. Lane, H. C., J. M. Depper, G. Whalen, T. A. Waldman, and A. S. Fauci. 1985. Qualitative analysis of immune function in patients with the acquired immunodeficiency syndrome. N. Engl. J. Med. 313:79

163. Lasalle, J. M., P. J. Tolentino, G. J. Freeman, L. Nadler, and D. A. Hafler. 1992. Early signaling defects in human T cells anergized by T cell presentation of autoantigen. Journal of Experimental Medecine 176:177-186.

164. Lasky, L. A., G. Nakamura, D. H. Smith, C. Fennie, C. Shimasaki, E. Patzer, P. Berman, T. Gregory, and D. J. Capon. 1987. Delineation of a region of the human immunodeficiency virus type 1 gp120 glycoprotein critical for interaction with the CD4 receptor. Cell 50:975-985.

165. Laurence, J., A. S. Hodtsev, and D. N. Posnett. 1992. Superantigen implicated in dependence of HIV-1 replication in T cells on TcR V β expression. Nature 358:255-257.

166. Laurent-Crawford, A. G., B. Krust, S. Muller, Y. Rivière, M. A. Rey-Cuille, J. M. Bechet, L. Montagnier, and A. G. Hovanessian. 1991. The cytopathic effect of HIV is associated with apoptosis. Virology 185:829-39.

167. Lazaro, I., D. Naniche, N. Signoret, A. M. Bernard, D. Marguet, D. Klatzmann, T. Dragic, M. Alizon, and Q. Sattentau. 1994. Factors involved in entry of the human immunodeficiency virus type 1 into permissive cells: lack of evidence of a role for CD26. Journal of Virology 68:6535-6546.

168. Ledbetter, J. A., N. K. Tonks, E. H. Fisher, and E. A. Clark. 1988. CD45 regulates signal transduction and lymphocyte activation by specific association with receptor molecules on T or B cells. Proc. Natl. Acad. Sci. U. S. A. 85:8628-8632.

169. Lee, W. T. and E. S. Vitetta. 1992. Changes in expression of CD45R during the development of Th1 and Th2 cell lines. Eur. J. Immunol. 22:1455-1459.

170. Letourneur, F. and R. D. Klausner. 1992. Activation of T cells by a tyrosine kinase activation domain in the cytoplasmic tail of CD3 ε. Science 255:79-82.

171. Letourneur, F. and R. D. Klausner. 1992. A novel di-leucine motif and a tyrosine-based motif independently mediate lysosomal targeting and endocytosis of CD3 chains. Cell 69:1143-1157.

172. Levin, S. D., K. M. Abraham, S. J. Anderson, K. A. Forbush, and R. M. Perlmutter. 1993. The protein tyrosine kinase $p56^{lck}$ regulates thymocyte development independently of its interaction with CD4 and CD8 coreceptors. J. Exp. Med. 178:245-255.

173. Levin, S. D., S. J. Anderson, K. A. Forbush, and R. M. Perlmutter. 1993. A dominant negative transgene defines a role for $p56^{lck}$ in thymopoiesis. EMBO J. 12:1671-1680.

174. Lindemann, D., R. Wilhelm, P. Renard, A. Althage, R. Zinkernagel, and J. Mous. 1994. Severe immunodeficiency associated with a human immunodeficiency virus 1 NEF/3'-long terminal repeat transgene. J. Exp. Med. 179:797-807.

175. Linette, G. P., R. J. Hartzman, J. A. Ledbetter, and C. H. June. 1988. HIV-1 infected T cells show a selective signaling defect after pertubation of CD3/antigen receptor. Science 241:573-576.

176. Loh, C., C. Romeo, B. Seed, J. T. Bruder, U. Rapp, and A. Rao. 1994. Association of raf with the CD3 γ and δ chains of the T cell receptor-CD3 complex. J. Biol. Chem. 269:8817-8825.

177. Love, P. E., E. W. Shores, M. D. Johnson, M. L. Tremblay, E. J. Lee, A. Grinberg, S. P. Huang, A. Singer, and H. Westphal. 1993. T cell development in mice that lack the ζ chain of the T cell antigen receptor complex. Science 261:918-921.

178. Luciw, P. A., C. Cheng-Mayer, and J. A. Levy. 1987. Mutational analysis of the human immunodeficiency virus: the orf-B region down regulates virus replication. Proc. Natl. Acad. Sci. USA 84:1434-1438.

179. Luescher, I. F., E. Vivier, A. Layer, Mahiou.J., F. Godeau, B. Malissen, and P. Romero. 1995. CD8 modulation of T cell antigen -ligand interactions on living cytotoxic T lymphocytes. Nature 373:353-356.

180. Luo, K. and B. M. Sefton. 1990. Analysis of the sites in $p56^{lck}$ whose phosphorylation is induced by tetradecanoyl phorbol acetate. Oncogene 5:803-808.

181. Luo, K. and B. M. Sefton. 1990. Cross-linking of T-cell surface molecules CD4 and CD8 stimulates phosphorylation of the ick tyrosine protein kinase at the autophosphorylation site. Mol. Cell. Biol. 10:5305-5313.

182. Luria, S., I. Chambers, and P. Berg. 1991. Expression of the type 1 human immunodeficiency virus Nef protein in T cells prevents antigen receptor-mediated induction of interleukin 2 mRNA. Proc. Natl. Acad. Sci. U. S. A. 88:5326-5330.

183. Maddon, P. J., J. S. McDougal, P. R. Clapham, A. G. Dalgleish, S. Jamal, R. A. Weiss, and R. Axel. 1988. HIV infection does not require endocytosis of its receptor CD4. Cell 54:865-874.

184. Maddon, P. J., S. M. Molineaux, D. E. Maddon, K. A. Zimmerman, M. Godfrey, F. W. Alt, L. Chess, and R. Axel. 1987. Structure and expression of the human and mouse T4 genes. Proc. Natl. Acad. Sci. USA 84:9155-9159.

185. Malissen, M., A. Gillet, B. Rocha, J. Trucy, E. Vivier, C. Boyer, F. Kontgen, N. Brun, G. Mazza, E. Spanopoulou, D. Guy-Grand, and B. Malissen. 1993. T cell development in mice lacking the CD3 ζ/η gene. EMBO J. 12:4347-4355.

186. Mann, D. L., F. Lasane, M. Popovic, L. O. Arthur, W. G. Robey, W. A. Blattner, and M. J. Newman. 1987. HTLV-III large envelope protein (gp120) suppresses PHA-induced lymphocyte blastogenesis. J. Immunol. 138:2640

187. Manolios, N., F. Letourneur, J. S. Bonifacino, and R. D. Klausner. 1991. Pairwise, cooperative and inhibitory interactions describe the assembly and probable structure of the T-cell antigen receptor. EMBO J. 10:1643-1651.

188. Mariani, R. and J. Skowronski. 1993. CD4 down-regulation by *nef* alleles isolated from human immunodeficiency virus type 1-infected individuals. Proc. Natl. Acad. Sci. USA. 90 (12):5549-53.

189. Marrack, P., R. Enders, R. Shimonkevitz, A. Zlotnik, D. Dialynas, F. Fitch, and J. Kappler. 1983. The major histocompatibility complex-restricted antigen receptor on T cells. II Role of the L3T4 product. J. Exp. Med. 158:1077-1091.

190. Marth, J. D., D. B. Lewis, C. B. Wilson, M. E. Gearn, E. G. Krebs, and R. M. Perlmutter. 1987. Regulation of $pp56^{lck}$ during T cell activation: functional implications for the src-like protein tyrosine kinases. EMBO J. 6:2727-2734.

191. McFarland, E. D., T. R. Hurley, J. T. Pingel, B. M. Sefton, A. Shaw, and M. L. Thomas. 1993. Correlation between Src family member regulation by the protein-tyrosine-phosphatase CD45 and transmembrane signaling through the T-cell receptor. Proc. Natl. Acad. of. Sci. USA. 90:1402-1406.

192. Mercep, M., J. S. Bonifacino, P. Garcia-Morales, L. E. Samelson, R. D. Klausner, and J. D. Ashwell. 1988. T cell CD3-ζη heterodimer expression and coupling to phosphoinositide hydrolysis. Science 242:571-574.

193. Mercep, M., A. M. Weissman, S. J. Frank, R. D. Klausner, and J. D. Ashwell. 1989. Activation-driven programmed cell death and T cell receptor ζη expression. Science 246:1162-1165.

194. Meyaard, L., S. A. Otto, R. R. Jonker, M. J. Mijnster, R. P. M. Keet, and F. Miedema. 1992. Programmed cell death of T cells in HIV-1 infection. Science 257:217-219.

195. Micali, S. 1993. Mechanism for the T4 lymphopenia of AIDS. Proc. Natl. Acad. Sci. USA 90:10982-10983.

196. Miller, M. D., M. T. Warmerdam, I. Gaston, W. C. Greene, and M. B. Feinberg. 1994. The human immunodeficiency virus-1 Nef gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages. J. Exp. Med. 179:101-113.

197. Minami, Y., T. Kono, K. Yamada, N. Kobayashi, A. Kawahara, R. M. Permulter, and T. Taniguchi. 1993. Association of $p56^{lck}$ with IL-2 receptor β chain is critical for the IL-2-induced activation of $p56^{lck}$. EMBO 12:759-768.

198. Minami, Y., A. M. Weissman, L. E. Samelson, and R. D. Klausner. 1987. Building a multi chain receptor: synthesis, degradation, and assembly of the T cell antigen receptor. Proc. Natl. Acad. Sci. USA 84:2688-2692.

199. Mittler, R. S. and M. K. Hoffman. 1989. Synergism between HIV gp120 and gp120-specific antibody in blocking human T cell activation. Science 245:1380

200. Mittler, R. S., B. M. Rankin, and P. A. Kiener. 1991. Physical associations between CD45 and CD4 or CD8 occur as late activation events in antigen receptor-stimulated human T cells. J. Immunol. 147:3434-3440.

201. Moebius, U., L. K. Clayton, S. Abraham, A. Denier, J. J. Yunis, S. C. Harrison, and E. L. Reinherz. 1992. Human immunodeficiency virus gp120 binding C'C" ridge of CD4 domain 1 is also involved in interaction with class II major histocompatibility complex molecules. Proc. Natl. Acad. Sci. USA 89:12008-12012.

202. Moebius, U., P. Pallai, S. C. Harrison, and E. L. Reinherz. 1993. Delineation of an extended surface contact area on human CD4 involved in class II major complex binding. Proc. Natl. Acad. Sci. USA 90:8259-8263.

203. Molina, T. J., M. F. Bachmann, T. M. Kundig, R. M. Zinkernagel, and T. W. Mak. 1993. Peripheral T cells in mice lacking $p56^{lck}$ do not express significant antiviral effector functions. J. Immunol. 151:699-706.

204. Molina, T. J., K. Kishihara, D. P. Siderovski, W. van Ewijk, A. Narendran, E. Timms, A. Wakeham, C. J. Paige, K. U. Hartmann, and A. Veillette. 1992. Profound block in thymocyte development in mice lacking $p56^{lck}$. Nature 357:161-164.

205. Mombaerts, P., J. Iacomini, R. S. Jonhson, K. Herrup, S. Tonegawa, and V. E. Papaioannou. 1992. RAG-1 deficient mice have no mature B and T lymphocytes. Cell 68:869-877.

206. Munis, J. R., R. S. Kornbluth, J. C. Guatelli, and D. D. Richman. 1992. Ordered appearance of human immunodeficiency virus type 1 nucleic acids following high multiplicity infection of macrophages. Journal of General Virology 73:1899-1906.

207. Murti, K. G., P. S. Brown, L. Ratner, and J. V. Garcia. 1993. Highly localized tracks of human immunodeficiency virus type 1 Nef in the nucleus of cells of a human CD4+ T-cell line. Proc. Natl. Acad. Sci. U. S. A. 90:11895-11899.

208. Mustelin, T. and A. Altman. 1990. Dephosphorylation and activation of the T cell tyrosine kinase $pp56^{lck}$ by the leukocyte common antigen (CD45). Oncogene 5:809-813.

209. Mustelin, T., K. M. Coggeshall, and A. Altman. 1989. Rapid activation of the T-cell tyrosine protein kinase $pp56^{lck}$ by the CD45 phosphotyrosine phophatase. Proc. Natl. Acad. Sci. USA. 86:6302-6306.

210. Nada, S., M. Okada, A. MacAuley, J. A. Cooper, and H. Nakagawa. 1993. Cloning of a complementary DNA for a protein-tyrosine kinase that specifically phosphorylates a negative regulatory site of p60 c-src. Nature 351:69-72.

211. Newell, M. K., L. J. Haughn, C. R. Maroun, and M. H. Julius. 1990. Death of mature T cells by separate ligation of CD4 and the T-cell receptor for antigen. Nature 347:286-289.

212. Niederman, T. M., J. V. Garcia, W. R. Hastings, S. Luria, and L. Ratner. 1992. Human immunodeficiency virus type 1 Nef protein inhibits NF-κB induction in human T cells. J. Virol. 66:6213-6219.

213. Niederman, T. M., W. R. Hastings, S. Luria, J. C. Bandres, and L. Ratner. 1993. HIV-1 Nef protein inhibits the recruitment of AP-1 DNA-binding activity in human T-cells. Virology 194:338-344.

214. Niederman, T. M., B. J. Thielan, and L. Ratner. 1989. Human immunodeficiency virus type 1 negative factor is a transcriptional silencer. Proc. Natl. Acad. Sci. USA 86:1128-1132.

215. Nossal, G. J. 1994. Negative selection of lymphocytes. Cell 76:229-239.

216. O'Rourke, A. M., J. Rogers, and M. F. Mescher. 1990. Activated CD8 binding to class I protein mediated by the T-cell receptor results in signalling. Nature 346:187-189.

217. Ohno, H., T. Aoe, S. Taki, D. Kitamura, Y. Ishida, K. Rajewski, and T. Saito. 1993. Developmental and functional impairment of T cells in mice lacking CD3 ζ chains. EMBO J. 12:4357-4366.

218. Ostergaard, H. L., D. A. Shackelford, T. R. Hurley, P. Johnson, R. Hyman, B. M. Sefton, and I. S. Trowbridge. 1989. Expression of CD45 alters phosphorylation of the lck-encoded tyrosine proteine kinase in murine lymphoma T cell line. Proc. Natl. Acad. Sci. USA 86:8959-8963.

219. Ostergaard, H. L. and I. S. Trowbridge. 1990. Coclustering CD45 with CD4 or CD8 alters the phosphorylation and kinase activity of $p56^{lck}$. J. Exp. Med. 172:347-350.

220. Ostergaard, H. L. and I. S. Trowbridge. 1991. Negative regulation of CD45 protein tyrosine phosphatase activity by ionomycin in T cells. Science 253:1423-1425.

221. Owens, T., B. Fazekas de St-Groth, and J. F. A. P. Miller. 1987. Coaggregation of the T-cell receptor with CD4 and other T-cell surface molecules enhances T-ceell activation. Proc. Natl. Acad. Sci. USA 84:9209

222. Oyaizu, N., N. Chirmule, V. S. Kalyanaraman, W. W. Hall, R. A. Good, and S. Pahwa. 1990. Human immunodeficiency virus type 1 envelope glycoprotein gp120 produce immune defects in CD4+ T lymphocytes by inhibiting interleukin-2 mRNA. Proc. Natl. Acad. Sci. USA 87:2379

223. Padovan, E., C. Giachino, M. Cella, S. Valitutti, O. Acuto, and A. Lanzavecchia. 1995. Normal T lymphocytes can express two different T cell receptor β chains: implications for the mechanism of allelic exclusion. Journal of Experimental Medicine 181:1587-1591.

224. Pantaleo, G., C. Graziosi, J. F. Demarest, L. Butini, M. Montroni, C. H. Fox, J. M. Orenstein, D. P. Kotler, and A. S. Fauci. 1993. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. Nature 362:355-359.

225. Pantaleo, G., C. Graziosi, and A. S. Fauci. 1993. The immunopathogenesis of human immunodeficiency virus infection. N. Engl. J. Med. 328:327-335.

226. Park, D. J., H. W. Rho, and S. G. Rhee. 1991. CD3 stimulation causes phosphorylation of phospholipase C-γ1 on serine and tyrosine residues in a human T-cell line. Proc. Natl. Acad. Sci. USA. 88:5453-5456.

227. Pelchen-Matthews, A., I. Boulet, D. R. Littman, R. Fagard, and M. Marsh. 1992. The protein tyrosine kinase $p56^{lck}$ inhibits CD4 endocytosis by preventing entry of CD4 into coated pits. J. Cell Biol. 117:279-290.

228. Pelchen-Matthews, A., I. J. Parsons, and M. Marsh. 1993. Phorbol ester-induced downregulation of CD4 is a multistep process involving dissociation from $p56^{lck}$, increased association with clathrin-coated pits and altered endosomal sorting. J. Exp. Med. 178:1209-1222.

229. Peri, K. G., F. G. Gervais, R. Weil, D. Davidson, G. D. Gish, and A. Veillette. 1993. Interactions of the SH2 domain of lymphocyte-specific tyrosine protein kinase $p56^{lck}$ with phosphotyrosine-containing proteins. Oncogene 8:2765-2772.

230. Perlmutter, R. M. 1990. Translational regulation of the lymphocyte-specific protein tyrosine kinase $p56^{lck}$. Enzyme 44:214-224.

231. Piatier-Tonneau, D., L.-N. Gastinel, G. Moussy, B. Benichou, F. Amblard, P. Vaigot, and C. Auffray. 1991. Mutations in the D strand of the human CD4 V1 domain affect CD4 interactions with the human immunodeficiency virus envelope glycoprotein gp120 and HLA class II antigens similarly. Proc. Natl. Acad. Sci. USA 88:6858-6862.

232. Pingel, J. T. and M. Thomas. 1989. Evidence that the leukocyte commun Ag is required for Ag induced T cell proliferation. Cell 58:1055-1065.

233. Prasad, K. V., R. Kapeller, O. Janssen, H. Repke, J. S. Duke-Cohan, L. C. Cantley, and C. E. Rudd. 1993. Phosphatidylinositol (PI) 3-kinase and PI 4-kinase binding to the CD4-p56^{lck} complex: the p56^{lck} SH3 domain binds to PI 3-kinase but not PI 4-kinase. Mol. Cell Biol. 13:7708-7717.

234. Punt, J. A., J. L. Roberts, K. P. Kearse, and A. Singer. 1994. Stochiometry of the T cell antigen receptor (TCR) complex: each TCR/CD3 complex contains one TCRα, one TCRβ, and two CD3ε chains. J. Exp. Med. 180:587-593.

235. Qian, D., I. Griswold-Prenner, M. Rich Rosner, and F. W. Fitch. 1993. Multiple components of the T cell antigen receptor complex become tyrosine-phosphorylated upon activation. J. Biol. Chem. 268:4488-4493.

236. Rahemtulla, A., W. P. FunglLeung, M. W. Schilham, T. M. Kundig, S. R. Sambhara, A. Narendran, A. Arabian, A. Wakeham, C. J. Paige, R. M. Zinkernagel, R. G. Miller, and T. W. Mak. 1991. Normal development and function of CD8+ cells but markedly decreased helper cell activity in mice lacking CD4. Nature 353:180-184.

237. Rebai, N., G. Pantaleo, J. F. Demarest, C. Ciurli, H. Soudeyns, J. W. Adelsberger, M. Vaccarezza, R. E. Walker, R. P. Sekaly, and A. S. Fauci. 1994. Analysis of the T cell receptor β chain variable region (Vβ) repertoire in monozygotic twins discordant for human immunodeficiency virus: evidence for pertubations of specific Vβ segments in CD4 positive T cells of the virus positive twins. Proc. Natl. Acad. Sci. USA 91:1529-1533.

238. Reed, J. C., J. D. Alpers, P. C. Nowell, and R. G. Hoover. 1986. Sequential expression of protooncogenes during lectin-stimulated mitogenesis of normal human lymphocytes. Proc. Natl. Acad. Sci. USA 83:3982-3986.

239. Reinherz, E. L. and S. F. Schlossman. 1980. The differentiation and function of human T lymphocytes. Cell 19:821-827.

240. Reiser, H., G. J. Freeman, Z. Razi-Wolf, C. D. Gimmi, B. Benacerraf, and L. M. Nadler. 1992. Murine B7 antigen provides an efficient costimulatory signal for activation of murine T lymphocytes via the T-cell receptor/CD3 complex. Proc. Natl. Acad. Sci. USA 89:271-275.

241. Rhee, S. S. and J. W. Marsh. 1994. HIV-1 Nef activity in murine T cells. CD4 modulation and positive enhancement. J. Immunol. 152:5128-5134.

242. Rhee, S. S. and J. W. Marsh. 1994. Human immunodeficiency virus type 1 Nef-induced down-modulation of CD4 is due to rapid internalization and degradation of surface CD4. J. Virol. 68:5156-5163.

243. Rivas, A., S. Takada, J. Koide, G. Sonderstrup-McDevitt, and E. G. Engleman. 1988. CD4 molecules are associated with the antigen receptor complex on activated but not resting T cells. J. Immunol. 140:2912-2918.

244. Robert-Guroff, M., M. Popovic, S. Gartner, P. Markham, R. C. Gallo, and M. S. Reitz. 1990. Structure and expression of tat-, rev-, and nef-specific transcripts of human immunodeficiency virus type 1 in infected lymphocytes and macrophages. Journa. of Virology 64:3391-3398.

245. Rojo, J. M., K. Saizawa, and C. A. Janeway. 1989. Physical association of CD4 and T cell can be induced by anti-T cell receptor antibodies. Proc. Natl. Acad. Sci. USA 86:3311.

246. Romeo, C., M. Amiot, and B. Seed. 1992. Sequence requirements for induction of cytolysis by the T cell Antigen /Fc receptor ζ chain. Cell 68:889-897.

247. Romeo, C. and B. Seed. 1991. Cellular immunity to HIV activated by CD4 fused to T cell or Fc receptor polypeptides. Cell 64:1037-1046.

248. Rosoff, P. M., S. J. Burakoff, and J. L. Greenstein. 1987. The role of L3T4 molecule in mitogen and antigen activated signal transduction. Cell 49:845-853.

249. Ross, S. E., B. Schraven, F. D. Goldman, J. Crabtree, and G. A. Koretzky. 1994. The association between CD45 and lck does not require CD4 or CD8 and is independent of T cell receptor stimulation. Biochem. Biophys. Res. Commun. 198:88-96.

250. Rudd, C. E., J. M. Trevillyan, J. V. Dasgupta, L. L. Wong, and S. F. Schlossman. 1988. The CD4 antigen is complexed in detergent lysates to a protein tyrosine kinase (pp58) from human T lymphocytes. Proc. Natl. Acad. Sci. USA 85:5190

251. Ruppert, J., J. Alexander, K. Snoke, M. Coggeshall, E. Herbert, D. McKenzie, H. M. Grey, and A. Sette. 1993. Effect of T cell receptor antagonism on interaction between T cells and antigen presenting cells and on T cell signaling events. Proc. Natl. Acad. Sci. USA 90:2671-2675.

252. Ryu, S., P. D. Kwong, A. Truneh, T. G. Porter, J. Arthos, M. Rosenberg, X. Dai, N. Xuong, R. Axel, R. W. Sweet, and W. A. Hendrickson. 1990. Crystal structure of an HIV-binding recombinant fragment of human CD4. Nature 348:419-426.

253. Saint-Ruf, C., K. Ungewiss, M. Groettrup, L. Bruno, H. J. Fehling, and H. von Boehmer. 1994. Analysis and expression of a cloned pre-T cell receptor gene. Science 266:1208-1212.

254. Saizawa, K., J. Rojo, and C. A. J. Janeway. 1987. Evidence for a physical association of CD4 and the CD3:α/β T cell receptor. Nature 328:260-263.

255. Salmon, P., R. Olivier, Y. Riviere, E. Brisson, J.-C. Gluckman, M.-P. Kieny, and D. Klatzmann. 1988. Loss of CD4 membrane expression and CD4 mRNA during acute HIV replication. J. Exp. Med. 168:1953

256. Samelson, L. E., A. F. Phillips, E. T. Luong, and R. D. Klausner. 1990. Association of the fyn protein-tyrosine kinase with the T-cell antigen receptor. Proc. Natl. Acad. Sci. U. S. A. 87:4358-4362.

257. Sancho, J., J. A. Ledbetter, M. S. Choi, S. B. Kanner, J. P. Deans, and C. Terhorst. 1992. CD3-ζ surface expression is required for CD4-p56^{lck}-mediated upregulation of T cell antigen receptor-CD3 signaling in T cells. Journal of Biological Chemistry. 267:7871-7879.

258. Sanfridson, A., B. R. Cullen, and C. Doyle. 1994. The simian immunodeficiency virus Nef protein promotes degradation of CD4 in human T cells. J. Biol. Chem. 269:3917-3920.

259. Sattentau, Q. and R. A. Weiss. 1988. The CD4 antigen: physiological ligand and HIV receptor. Cell 52:631

260. Sawada, S. and D. R. Littman. 1991. Identification and characterization of a T-cell-specific enhancer adjacent to the murine CD4 gene. Mol. Cell. Biol. 11:5506-5515.

261. Sawada, S., J. D. Scarborough, N. Killeen, and D. R. Littman. 1994. A lineage-specific transcriptional silencer regulates CD4 gene expression during T lymphocyte development. Cell 77:917-929.

262. Schafer, P. H. and S. K. Pierce. 1994. Evidence for dimers of MHC class II molecules in B lymphocytes and their role in low affinity T cell responses. Immunity 1:699-707.

263. Schatz, D. G., M. A. Oettinger, and D. Baltimore. 1989. The V(D)J recombination activating gene, RAG-1. Cell 59:10-35-1048.

264. Schatz, D. G., M. A. Oettinger, and M. S. Schlissel. 1992. V(D)J recombination: molecular biology and regulation. Ann. Rev. Immunol. 10:359-383.

265. Schraven, B., H. Kirchgessner, B. Gaber, Y. Samstag, and S. Meuer. 1991. A functional complex is formed in human T lymphocytes between the protein tyrosine phosphatase CD45, the protein tyrosine kinase $p56^{lck}$ and pp32, a possible common substrate. Eur. J. Immunol. 21:2469-2477.

266. Schraven, B., Y. Samstag, P. Altevogt, and S. C. Meuer. 1990. Association of CD2 and CD45 on human T lymphocytes. Nature 345:71-74.

267. Schwartz, O., M. Alizon, J. M. Heard, and O. Danos. 1994. Impairment of T cell receptor-dependent stimulation in CD4+ lymphocytes after contact with membrane-bound HIV-1 envelope glycoprotein. Virology 198:360-365.

268. Schwartz, O., A. Dautry-Varsat, B. Goud, V. Marechal, A. Subtil, J. M. Heard, and O. Danos. 1995. Human immunodeficiency virus type 1 Nef induces accumulation of CD4 in early endosomes. J. Virol. 69:528-533.

269. Schwartz, R. H. 1990. A cell culture model for T lymphocyte clonal anergy. Science 248:1349-1356.

270. Schwinzer, R., R. A. Franklin, J. Domenico, H. Renz, and E. W. Gelfand. 1992. Monoclonal antibodies directed to different epitopes in the TcR-CD3 complex induce different states of competence in resting human T cells. J. Immunol. 148:1322-1328.

271. Shaw, A. S., K. E. Amrein, C. Hammond, D. F. Stern, B. M. Sefton, and J. K. Rose. 1989. The cytoplasmic domain of CD4 interacts with the tyrosine protein kinase, $p56^{lck}$, through its unique amino-terminal domain. Cell 59:627

272. Shaw, A. S., J. Chalupny, A. Whitney, C. Hammond, K. E. Amrein, P. Kavathas, B. M. Sefton, and J. K. Rose. 1990. Short related sequences in the cytoplasmic domains of CD4 and CD8 mediated binding to the amino-terminal domain of the $p56^{lck}$ tyrosine protein kinase. Mol. Cell. Biol. 10:1853-1862.

273. Shenoy-Scaria, A. M., J. Kwong, T. Fujita, M. W. Olszowy, A. S. Shaw, and D. M. Lublin. 1992. Signal transduction through decay-accelerating factor. J. Immunol. 149:3535-3541.

274. Shenoy-Scaria, A. M., L. K. Timson Gauen, J. Kwong, A. S. Shaw, and D. M. Lublin. 1993. Palmitylation of an amino-terminal cysteine motif of protein tyrosine kinases $p56^{lck}$ and $p59^{fyn}$ mediates interaction with glycosyl-phosphatidylinositol-anchored proteins. Mol. Cell. Biol. 13:6385-6392.

275. Shin, J., C. Doyle, Z. Yang, D. Kappes, and J. Strominger. 1990. Structural features of the cytoplasmic region of CD4 required for internalization. EMBO Journal 9:425-434.

276. Shin, J., R. L. J. Dunbrack, S. Lee, and J. L. Strominger. 1991. Phosphorylation-dependent down-modulation of CD4 requires a specific structure within the cytoplasmic domain of CD4. J. Biol. Chem. 266:10658-10665.

277. Shinkai, Y., S. Koyasu, K. Nakayama, K. M. Murphy, D. Y. Loh, E. L. Reinherz, and F. W. Alt. 1993. Restoration of T cell development in RAG-2-deficient mice by functional TCR transgenes. Science 259:822-825.

278. Shiroo, M., L. Goff, M. Biffen, E. Shivnan, and D. Alexander. 1992. CD45 tyrosine phosphatase-activated $p59^{fyn}$ couples the T cell antigen receptor to pathways of diacylglycerol production,protein kinase C activation and calcium influx. EMBO J. 11:4887-4897.

279. Shores, E. W., K. Huang, T. Tran, E. J. Lee, A. Grinberg, and P. E. Love. 1994. Role of the TcR ζ chain in T cell development and selection. Science 266:1047-1050.

280. Sieh, M., J. B. Bolen, and A. Weiss. 1993. CD45 specifically modulates binding of Lck to a phosphopeptide encompassing the negative regulatory tyrosine of Lck. EMBO J. 12:315-321.

281. Skowronski, J., D. Parks, and R. Mariani. 1993. Altered T cell activation and development in transgenic mice expressing the HIV-1 *nef* gene. EMBO J. 12:703-713.

282. Sleckman, B. P., A. Peterson, W. K. Jones, J. A. Foran, J. L. Greenstein, B. Seed, and S. J. Burakoff. 1987. Expression and function of CD4 in a murine T-cell hybridoma. Nature 328:351-353.

283. Sleckman, B. P., J. Shin, V. E. Igras, T. L. Collins, J. L. Strominger, and S. J. Burakoff. 1992. Disruption of the CD4-$p56^{lck}$ complex is required for rapid internalization of CD4. Proc. Natl. Acad. Sci. USA 89:7566-7570.

284. Sloan-Lancaster, J., B. D. Evavold, and P. M. Allen. 1993. Induction of T cell anergy by altered T-cell receptor ligand on live antigen-presenting cells. Nature 363:156-159.

285. Sloan-Lancaster, J., A. S. Shaw, J. B. Rothbard, and P. M. Allen. 1994. Partial T cell signaling: altered phospho-ζ and lack of ZAP-70 recruitment in APL-induced T cell anergy. Cell 79:913-922.

286. Soula, M., R. Fagard, and S. Fisher. 1992. Interaction of human immunodeficiency virus glycoprotein gp160 with CD4 in jurkat cells increases $p56^{lck}$ autophosphorylation and kinase activity. Int. Immunol. 4:295

287. Soula, M., B. Rothhut, L. Camoin, J.-L. Guillaume, D. Strosberg, T. Vorherr, P. Burn, F. Meggio, S. Fisher, and R. Fagard. 1993. Anti-CD3 and phorbol ester induce distinct phosphorylated sites in the SH2 domain of $p56^{lck}$. J. Biol. Chem. 268:27420-27427.

288. Spina, C. A., T. J. Kwoh, M. Y. Chowers, J. C. Guatelli, and D. D. Richman. 1994. The importance of *nef* in the induction of human immunodeficiency virus type 1 replication from primary quiescent CD4 lymphocytes. J. Exp. Med. 179:115-123.

289. Stanley, J. B., R. Gorczynski, C. Huang, J. Love, and G. B. Mills. 1990. Tyrosine phosphorylation is an obligatory event in Il-2 secretion. J. Immunol. 145:2189-2198.

290. Stefanova, I., V. Horejsi, I. J. Ansotegui, W. Knapp, and H. Stockinger. 1991. GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. Science 254:1016-1019.

291. Stein, B. S., S. D. Gowda, J. D. Lifson, R. C. Penhallow, K. G. Bensch, and E. G. Engleman. 1987. pH-independent HIV entry into CD4-positive T cells via virus envelope fusion to the plasma membrane. Cell 49:659-668.

292. Stein, P. L., H. M. Lee, S. Rich, and P. Soriano. 1992. $pp59^{fyn}$ mutant mice display differential signaling in thymocytes and peripheral T cells. Cell 70:741-750.

293. Stover, D. R., H. Charbonneau, N. K. Tonks, and K. A. Walsh. 1991. Protein-tyrosine-phosphatase CD45 is phosphorylated transiently on tyrosine upon activation of Jurkat T cells. Proceedings. of. the. National. Acad. Sci. USA. 88:7704-7707.

294. Straus, D. B. and A. Weiss. 1993. The CD3 chains of the T cell antigen receptor associate with the ZAP-70 tyrosine kinase and are tyrosine phosphorylated after receptor stimulation. J. Exp. Med. 178:1523-1530.

295. Strauss, B. and A. Weiss. 1992. Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor. Cell 70:585-593.

296. Swain, S. L. 1983. T cell subsets and the recognition of MHC Class. Immunol. Rev. 74:129

297. Taichman, R., I. Merida, T. Torigoe, G. N. Gaulton, and J. C. Reed. 1993. Evidence that protein tyrosine kinase p56-Lck regulates the activity of phosphatidylinositol-3'-kinase in interleukin-2-dependent T-cells. J. Biol. Chem. 268:20031-20036.

298. Terwilliger, E. F., E. Langhoff, D. Gabuzda, E. Zazopoulos, and W. A. Haseltine. 1991. Allelic variation in the effects of the *nef* gene on replication of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA. 88:10971-10975.

299. Thomas, P. M. and L. E. Samelson. 1992. The glycophosphatidylinositol-anchored Thy-1 molecule interacts with the $p60^{fyn}$ protein tyrosine kinase in T cells. J. Biol. Chem. 267:12317-12322.

300. Thome, M., P. Duplay, M. Guttinger, and O. Acuto. 1995. Syk and ZAP-70 mediates recruitement of $p56^{lck}$/CD4 to the activated T cell receptor/CD3/zeta complex. J. Exp. Med. 181:1997-2006.

301. Timson Gauen, L. K., Y. Zhu, F. Letourneur, Q. Hu, J. B. Bolen, L. A. Matis, R. D. Klausner, and A. S. Shaw. 1994. Interactions of $p59^{fyn}$ and ZAP-70 with T cell receptor activation motifs: defining the nature of a signalling motif. Mol. Cell. Biol. 14:3729-3741.

302. Tite, J. P., A. Sloan, and C. A. Janeway. 1986. The role of L3T4 in T-cell activation: L3T4 may be both an Ia binding protean and receptor that transduces a negative signal. J. Mol. Cell. Immunol. 2:179

303. Torigoe, T., J. A. Millan, K. W. Chan, R. Tachman, A. A. Brian, and J. C. Reed. 1994. Protein tyrosine kinase $p56^{lck}$ regulates lymphocyte function-associated 1 adhesion molecule expression, granule exocytosis, and cytolytic effector function in a cloned T cell. J. Exp. Med. 180:1115-1127.

304. Trowbridge, I. S. 1991. CD45. A prototype for transmembrane protein tyrosine phosphatases. Journal of Biological Chemistry. 266:23517-23520.

305. Truneh, A., D. Buck, D. R. Cassatt, R. Juszczak, S. Kassis, S. E. Ryu, D. Healey, R. Sweet, and Q. Sattentau. 1991. A region in domain 1 of CD4 distinct from the primary gp120 binding site is involved in HIV infection and virus-mediated fusion. Journal of Biological Chemistry. 266:5942-5948.

306. Turner, J. M., M. H. Brodsky, B. A. Irving, S. D. Levin, R. M. Perlmutter, and D. R. Littman. 1990. Interaction of the unique N-terminal region of tyrosine kinase p56^{lck} with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. Cell 60:755-765.

307. Ullrich, A. and J. Schlessinger. 1990. Signal transduction by receptors with tyrosine kinase activity. Cell 61:203-212.

308. Vaishnav, Y. N. and F. Wong-Staal. 1991. The biochemistry of AIDS. Annu. Rev. Biochem. 60:577-630.

309. Valentine, M. A., M. B. Widmer, J. A. Ledbetter, F. Pinault, R. Voice, E. A. Clark, B. Gallis, and D. L. Brautigan. 1991. Interleukin-2 stimulates serine phosphorylation of CD45 in CTLL-2.4 cells. Eur. J. Immunol. 21:913-919.

310. Valitutti, S., S. Muller, M. Cella, E. Padovan, and A. Lanzavecchia. 1995. Serial trigering of many T cell receptors by a few peptide-MHC complexes. Nature 375:148-150.

311. Veillette, A., M. A. Bookman, E. M. Horak, and J. B. Bolen. 1988. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56^{lck}. Cell 55:301-308.

312. Veillette, A., M. A. Bookman, E. M. Horak, L. E. Samelson, and J. B. Bolen. 1989. Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase p56^{lck}. Nature 338:257

313. Veillette, A., L. Caron, M. Fournel, and T. Pawson. 1992. Regulation of the enzymatic function of the lymphocyte-specific tyrosine protein kinase p56^{lck} by the non-catalytic SH2 and SH3 domains. Oncogene 7:971-980.

314. Veillette, A., I. D. Horak, E. M. Horak, M. A. Bookman, and J. B. Bolen. 1988. Alterations of the lymphocyte-specific protein tyrosine kinase (p56^{lck}) during T-cell activation. Mol. Cell. Biol. 8:4343-4361.

315. Vignali, D. A. and J. L. Strominger. 1994. Amino acid residues that flank core peptide epitopes and the extracellular domains of CD4 modulate differential signaling through the T cell receptor. Journal of Experimental Medicine 179:1945-1956.

316. Volarevic, S., B. B. Niklinska, C. M. Burns, H. Yamada, C. H. June, F. J. Dumont, and J. Ashwell. 1992. The CD45 tyrosine phosphatase regulates phosphotyrosine homeostasis and its loss reveals a novel pattern of late T cell receptor-induced Ca2+ oscillations. J Exp Med 176:835-844.

317. Volaveric, S., C. Burns, J. J. Sussman, and J. D. Ashwell. 1990. Intimate association of Thy-1 and the T cell antigen receptor with the CD45 tyrosine phosphatase. Proc. Natl. Acad. Sci. USA 87:7085-7089.

318. Volaveric, S., B. B. Niklinska, C. Burns, C. H. June, A. M. Weissman, and J. D. Ashwell. 1993. Regulation of TcR signaling by CD45 lacking transmembrane and extracellular domains. Science 260:541-544.

319. von Boehmer, H. 1994. Positive selection of lymphocytes. Cell 76:219-228.

320. Wang, J., Y. Yan, T. P. J. Garrett, J. Liu, D. W. Rodgers, R. L. Garlick, G. E. Tarr, Y. Husain, E. L. Reinherz, and S. C. Harrison. 1990. Atomic structure of a fragment of human CD4 containing two immunoglobulin-like domains. Nature 348:411-418.

321. Wange, R. L., A. N. Kong, and L. E. Samelson. 1992. A tyrosine phosphorylated 70kd protein binds a photoaffinity analogue of ATP and associates with both the ζ chain and CD3 components of activated T cell antigen receptor. J. Biol. Chem. 267:11685-11688.

322. Wange, R. L., S. N. Malek, S. Desiderio, and L. E. Samelson. 1993. Tandem SH2 domains of ZAP-70 bind to T cell antigen receptor ζ and CD3 ε from activated Jurkat T cells. Journal of Biological Chemistry. 268:19797-19801.

323. Weber, J. R., G. M. Bell, M. Y. Han, T. Pawson, and J. B. Imboden. 1992. Association of the tyrosine kinase lck with phospholipase C-γ1 after stimulation of the T cell antigen receptor. J. Exp. Med. 176:373-379.

324. Wegener, A. M., F. Letourneur, A. Hoeveler, T. Brocker, F. Luton, and B. Malissen. 1992. The T cell receptor/CD3 complex is composed of at least two autonomous transduction modules. Cell 68:83-95.

325. Wei, X., S. K. Ghosh, M. E. Taylor, V. A. Johnson, E. A. Emini, P. Deutsch, J. D. Lifson, S. Bonhoeffer, M. A. Nowak, and B. H. Hahn. 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature 373:117-122.

326. Weil, R., J. F. Cloutier, M. Fournel, and A. Veillette. 1995. Regulation of ZAP-70 by src family tyrosine protein kinases in an antigen specific T cell line. J. Biol. Chem. 270:2791-2799.

327. Weil, R. and A. Veillette. 1994. Intramolecular and extramolecular mechanisms repress the catalytic function of $p56^{lck}$ in resting T-lymphocytes. J. Biol. Chem. 269:22830-22838.

328. Westendorp, M. O., R. Frank, C. Ochsenbauer, K. Stricker, J. Dhein, H. Walczak, K. M. Debatin, and P. H. Krammer. 1995. Sensitization of T cells to CD95 mediated apoptosis by HIV-1 Tat and gp120. Nature 375:497-500.

329. Weyand, C. M., J. Goronzy, and G. Fathman. 1987. Modulation of CD4 by antigenic activation. J. Immunol 138:1351-1354.

330. Wilde, D. B., P. Marrack, J. Kappler, D. P. Dialynas, and F. W. Fitch. 1983. Evidence implicating L3T4 in class II MHC antigen reactivity; monoclonal antibody GK1.5 (anti-L3T4a) blocks class II MHC antigen-specific proliferation, release of lymphokines, and binding by cloned murine helper T lymphocyte lines. J. Immunol. 131:2178-2183.

331. Wildin, R. S., A. M. Garvin, S. Pawar, D. B. Lewis, K. M. Abraham, K. A. Forbush, S. F. Ziegler, J. M. Allen, and R. M. Perlmutter. 1991. Developmental regulation of lck gene expression in T lymphocytes. J. Exp. Med. 173:383-393.

332. Willey, R. L., F. Maldarelli, M. A. Martin, and K. Strebel. 1992. Human immunodeficiency virus type 1 Vpu protein induces rapid degradation of CD4. J. Virol. 68:7193-7200.

333. Winkler, D. G., I. Park, T. Kim, N. S. Payne, C. T. Walsh, J. L. Strominger, and J. Shin. 1993. Phosphorylation of Ser-42 and Ser-59 in the N-terminal region of the tyrosine kinase $p56^{lck}$. Proc. Natl. Acad. Sci. USA 90:5176-5180.

334. Xu, H. and D. R. Littman. 1993. A kinase-independent function of Lck in potentiating antigen-specific T cell activation. Cell 74:633-643.

335. Yao, X.-J., J. Friborg, F. Checroune, S. Gratton, F. Boisvert, R.-P. Sekaly, and E. A. Cohen. 1995. Degradation of CD4 induced by human immunodeficiency virus type 1 vpu protein: a predicted α-helical structure in the proximal cytoplasmic region of CD4 contributes to vpu sensitivity. Virology 209:615-623.

336. Zazopoulos, E. and W. A. Haseltine. 1992. Mutational analysis of the human immunodeficiency virus type 1 Eli Nef function. Proc. Natl. Acad. Sci. U. S. A. 89:6634-6638.

337. Zazopoulos, E. and W. A. Haseltine. 1993. Effect of *nef* alleles on replication of human immunodeficiency virus type 1. Virology 194:20-27.

338. Zerbib, A. C., A. B. Reske-Kunz, P. Lock, and R. P. Sekaly. 1994. CD4-mediated enhancement or inhibition of T cell activation does not require the CD4:$p56^{lck}$ association. Journal of Experimental Medicine 179:1973-1983.

339. Zijlstra, M., M. Bix, N. E. Simister, J. M. Loring, D. H. Raulet, and R. Jaenisch. 1990. β2-microglobulin deficient mice lack CD4-8+ cytolitic T cells. Nature 344:742-746.

CHAPTER 2

ASSOCIATION OF TYROSINE KINASE $p56^{lck}$ WITH CD4 INHIBITS THE INDUCTION OF GROWTH THROUGH THE αβ T CELL RECEPTOR

Association of tyrosine kinase $p56^{lck}$ with CD4 inhibits the induction of growth through the $\alpha\beta$ T-cell receptor

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THE membrane glycoprotein CD4 enhances antigen-mediated activation of T cells restricted by class II molecules of the major histocompatibility complex (MHC)[1–3]. This positive function has been attributed to the protein tyrosine kinase $p56^{lck}$ (ref. 4), which is noncovalently associated with the cytoplasmic portion of CD4[5,6], and is activated on CD4 aggregation[7]. Antigen presentation by MHC class II molecules coaggregates CD4 and the T-cell antigen receptor (TCR$\alpha\beta$–CD3)[8]. Thus, the mutual specificity of CD4 and TCR$\alpha\beta$ for the MHC–antigen complex results in the juxtaposition of $p56^{lck}$ and TCR$\alpha\beta$–$CD3^{9-13}$. In contrast, anti-CD4 antibodies can abrogate antigen-induced[14–17], as well as anti-TCR-induced[18–20] T-cell activation, indicating that CD4 might also transduce negative signals. The molecular basis for this opposing function remains unclear. Here we show that the CD4–$p56^{lck}$ complex prohibits the induction of activation signals through the TCR–CD3 complex when not specifically included in the signalling process. This negative effect does not require anti-CD4 treatment, indicating that the induction of distinct negative signals is probably not involved. Rather, the results demonstrate that the CD4–$p56^{lck}$ complex provides prerequisite signals for antigen-receptor-induced T-cell growth and thus characterize a molecular mechanism for functional constraints imposed on T-cell activation by the MHC.

We have isolated $CD4^+$ and $CD4^-$ variants of a $V\beta4$-expressing T-cell clone specific for an ovalbumin-derived peptide in association with $I\text{-}A^b$ (ref. 21). The $CD4^+$ variant clone 2.5 and the $CD4^-$ variant clone 2.10 respond comparably to a broad range of antigen concentration (10–100 $\mu g\ ml^{-1}$). Thus, CD4 neither provides a prerequisite signal, nor needs to function as an adhesion molecule[15,17] in the antigen-specific response of these cells. Nonetheless, antigen-induced growth of clone 2.5 is inhibited by anti-CD4 antibodies (Table 1*a*), indicating that antibody-induced aggregation of CD4 may deliver a negative signal[18–20]. The surprising observation came when assessing the responsiveness of clones 2.5 and 2.10 to anti-TCR$\alpha\beta$ (H57.597, ref. 22), and anti-V$\beta4$ (KT4.10, ref. 23) monoclonal antibodies. Although both clones respond comparably to anti-CD3ε (145.2C11, ref. 24), the responses of the $CD4^+$ clone 2.5 to either TCR$\alpha\beta$- or V$\beta4$-specific antibodies were more than 10-fold lower than those observed with the $CD4^-$ clone 2.10 (Table 1*a*). Because both clones express functional TCR–CD3 complexes (Table 1*a*), these results suggest that expression of CD4 by clone 2.5 may be responsible for its inability to respond to the TCR-specific antibodies.

To investigate this, we infected clone 2.10 with retroviruses containing the neomycin resistance gene alone, or in combination with mouse complementary DNAs encoding either wild-type CD4 or a mutated CD4 molecule in which the cysteine residues required for association with the cellular tyrosine kinase $p56^{lck}$ (refs 25, 26) were substituted with alanine residues. Figure 1*a* illustrates that infection with retrovirus encoding either the double cysteine CD4 mutant (NDC) or wild-type CD4 (NC) resulted in stable levels of CD4 expression ranging from 65% to 100% of that observed on clone 2.5. Lck immunoblots reveal that these clones contain comparable levels of $p56^{lck}$ (Fig. 1*b*). Further, anti-CD4 coprecipitated comparable levels of $p56^{lck}$ from clone 2.5 and wild-type CD4 infectants of clone 2.10 (Fig. 1*b*). As previously reported[25,26], $p56^{lck}$ did not coprecipitate with CD4 containing the alanine substitutions.

Expression of the neomycin resistance gene in clone 2.10

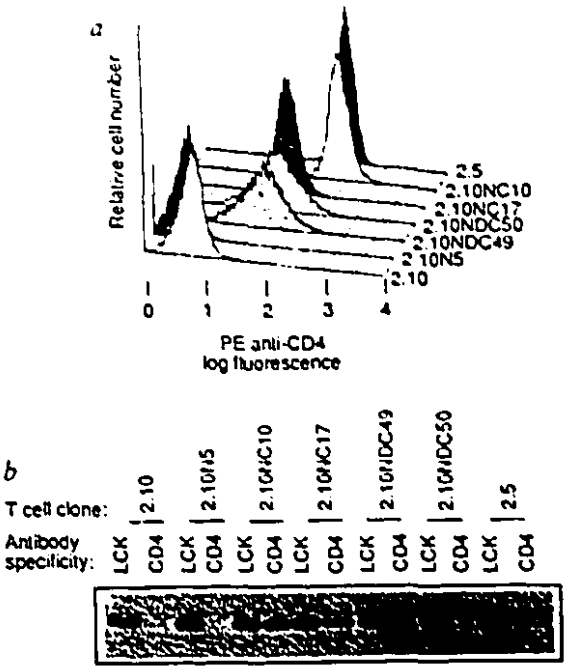

FIG. 1 *a*, Expression of exogenous CD4 in clone 2.10. *b*, Differential coprecipitation of $p56^{lck}$ with CD4 variants. *a*, Wild-type mouse CD4 cDNA (provided by J. Parnes, Stanford University, Palo Alto, California) was subcloned into the retrovirus expression vector, pLXSN (ref. 41). A mutated mouse CD4 cDNA, containing alanine substitutions at residues 418 and 420 was generated using the polymerase chain reaction (PCR) overlap-extension technique as previously described[42] and subcloned into the retroviral expression vector, MNC Stuffer[43]. Both pLXSN and MNC Stuffer contain the neomycin resistance gene (*neo*). DAMP cells[43] were transfected with MNC Stuffer containing the mutated CD4 cDNA, and Ψ-2 cells[44] were transfected with either pLXSN containing no insert or pLXSN containing wild-type CD4 cDNA. Subsequently, where applicable, drug-resistant packaging cells were sorted for high surface expression of CD4. Clone 2.10 was infected with virus containing *neo* (N), *neo*-wild-type CD4 (NC), or *neo*-mutated CD4 (NDC), using either supernatants of sorted, transfected packaging cells[45], or by coculture with mitomycin C-treated, sorted, transfected packaging cells. $CD4^+$ infectants were enriched for high-level CD4 expression by cell sorting, and then cloned by micromanipulation or FACSTAR autoclone. Infectants were maintained in medium supplemented with 8 U ml^{-1} rIL-2 and 500 $\mu g\ ml^{-1}$ G418. CD4 expression by clone 2.10, *neo*-infectants (N), *neo*-wild-type CD4 infectants (NC), and *neo*-mutated CD4 infectants (NDC) of clone 2.10, as well as clone 2.5, was determined by immunofluorescent staining and FACS analysis using phycoerythrin (PE) conjugated anti-CD4 (GK1.5 Becton Dickinson). *b*, Lck immunoblots were done as previously described[6]. Briefly, T cells were lysed at 5×10^7 cells ml^{-1} in lysis buffer containing 1% Nonidet P-40, and postnuclear fractions were prepared by spinning lysates at 13,000*g* for 10 min. CD4 was precipitated from 5×10^6 cell equivalents using 1 μg anti-CD4 (H129, ref. 37). Immune complexes were subsequently collected by the addition of *Staphylococcus aureus* (Staph A, Pansorbin, Calbiochem), which had been pre-coated with polyclonal rabbit anti-rat antiserum (RAR, Jackson). Lck was precipitated directly by adding 1 μl rabbit anti-$p56^{lck}$ (ref. 5) to lysates containing 5×10^6 cell equivalents, and immune complexes collected with Staph A. CD4 and $p56^{lck}$ precipitates were fractionated by SDS–PAGE (8% acrylamide), and transferred to nitrocellulose, blocked, incubated with rabbit anti-$p56^{lck}$, and developed with ^{125}I-labelled protein A.

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infectants did not alter responsiveness to antigen, anti-TCR$\alpha\beta$, or anti-CD3ε (Table 1, Fig. 2*a–c*). In contrast, the responses to TCR$\alpha\beta$ antibody by all wild-type CD4 infectants of clone 2.10 were virtually abrogated (Fig. 2*e*), a phenotype identical to that observed in the CD4$^-$ clone 2.5 (Table 1). Moreover, as observed with clone 2.5, these wild-type CD4 infectants responded well to both antigen (Fig. 2*d*) and anti-CD3ε (Fig. 2*f*), indicating that the TCR-CD3 complex was indeed functional.

To determine directly whether CD4 expression *per se* is sufficient to prohibit the induction of growth by TCR$\alpha\beta$ antibody, we analysed the response of mutated CD4 infectants of clone 2.10 (NDC) to TCR$\alpha\beta$ antibody. As illustrated in Fig. 2*h*, despite expression levels of CD4 comparable to wild-type CD4 infectants of clone 2.10 (Fig. 2*j*), mutated CD4 infectants responded as well to anti-TCR$\alpha\beta$ as they did to antigen (Fig. 2*g*). To emphasize the point, proliferation results obtained from each category of infectant were normalized by creating a ratio of the responses of each clone to TCR$\alpha\beta$ antibody and to antigen. As illustrated in Fig. 2*j*, plotting this ratio relative to the level of CD4 expression for each clone reveals that both control (N) and mutated CD4 (NDC) infectants of clone 2.10 respond as well to TCR$\alpha\beta$ antibody as they do to antigen. In contrast, responses of wild-type CD4 (NC) infectants to anti-TCR$\alpha\beta$ range from 1 to 15% of those obtained with antigen. Thus, the capacity of CD4 to prohibit the induction of T-cell growth by TCR$\alpha\beta$ antibody correlates with its association with p56^{lck}.

As suggested by many studies, antigen presented in association with MHC class II molecules would serve to coaggregate CD4 and TCR$\alpha\beta$-CD3, juxtaposing CD4-associated p56^{lck} and its putatively relevant substrates[1–3,10–13]. The prediction follows that deliberate antibody-mediated coaggregation of CD4 and TCR-CD3 in clone 2.5 and in wild-type CD4 infectants of clone 2.10 should result in the induction of T-cell growth, as is the case (Table 1*b*). Rat monoclonal antibody specific for either Vβ4, CD4, or a combination of the two were added to culture wells coated with polyclonal mouse anti-rat IgG. Clone 2.10

TABLE 1 Effects of CD4 on T-cell activation

(*a*) Expression of CD4 correlates with the failure of TCR$\alpha\beta$ antibodies to induce growth

	Thymidine incorporation (c.p.m. $\times 10^{-3}$) in response to				
Clone	OVA/I-A^b	OVA/I-A^b +anti-CD4	Anti-CD3ε	Anti-TCR$\alpha\beta$	Anti-Vβ4
2.5$^{CD4^-}$	61.3 ± 2.3	4.6 ± 0.2	72.0 ± 7.0	4.7 ± 0.2	3.1 ± 0.2
2.10$^{CD4^-}$	45.5 ± 2.6	41.4 ± 0.5	88.4 ± 7.6	59.7 ± 0.9	53.8 ± 2.0

(*b*) Induction of growth through TCR$\alpha\beta$ requires its coaggregation with CD4-p56^{lck}

	Thymidine incorporation (c.p.m. $\times 10^{-3}$) in response to			
Clone	Anti-CD4	Anti-Vβ4	Anti-CD4 +anti-Vβ4	Synergy
2.5	0.04 ± 0.005	0.29 ± 0.05	88.1 ± 2.9	266
2.10	0.03 ± 0.002	16.4 ± 1.1	19.4 ± 1.1	1.2
2.10NC10	0.03 ± 0.003	0.35 ± 0.05	96.1 ± 1.1	253
2.10NDC50	0.04 ± 0.006	127.7 ± 4.3	118.4 ± 4.7	0.93

a, Clones were maintained in serum-free Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 8 U ml^{-1} recombinant IL-2[28]. Before assay, cells were collected and washed twice in PBS containing 5% heat-inactivated fetal calf serum. T cells (5×10^4) were cultured, in the absence of IL-2, in 0.2 ml IMDM containing 5×10^5 irradiated (2,000 rads) syngeneic (C57BL/6) splenocytes. Cultures were stimulated with 100 μg ml^{-1} ovalbumin, in the presence or absence of 2 μg ml^{-1} anti-CD4 (H129, ref. 37). Anti-TCR$\alpha\beta$ (H57.597, ref. 22) or anti-CD3ε (145.2C11, ref. 24) were used at 1 μg ml^{-1}, and anti-Vβ4 (KT410.1, ref. 23) at 3 μg ml^{-1}. At 40 h, cultures received 1 μCi ^{3}H-TdR; 6 h later, they were collected onto filter mats and thymidine uptake assessed by liquid scintillation spectroscopy. Values represent the mean of five experiments, with one standard error indicated. Clones 2.5 and 2.10 responded to 100 μg ml^{-1} Keyhole Limpet Haemocyanin with 150 ± 15 c.p.m. and 120 ± 14 c.p.m., respectively. The addition of 2 μg ml^{-1} anti-MHC class II (M5114, ref. 38) inhibited the antigen response of both 2.5 and 2.10, resulting in 120 ± 10 c.p.m. and 74 ± 5 c.p.m., respectively. Neither anti-MHC Class I (M1/42, ref. 39) nor anti-CD8α (53.6.72, ref. 40) inhibited antigen responses. *b*, Culture plates were precoated for 1 h at 37 °C with 100 μl 50 μg ml^{-1} polyclonal mouse anti-rat antiserum (Jackson). Wells were washed 3–4 times with PBS and blocked for 1 h at 37 °C with a 5% solution of BSA (Fraction V Albumin, Boehringer Mannheim). Wells were then washed and incubated at 37 °C with IMDM containing 0.4 μg ml^{-1} anti-CD4 or 0.15 μg ml^{-1} anti-Vβ4, or a combination of the two. T cells were added (3.5×10^4 cells per well) and cultures incubated for 25–30 h at 37 °C before addition of 1 μCi ^{3}H-TdR per well. Six hours later, cultures were collected onto filter mats and thymidine uptake assessed by liquid scintillation spectroscopy. Values represent the mean of three experiments with one standard error indicated.

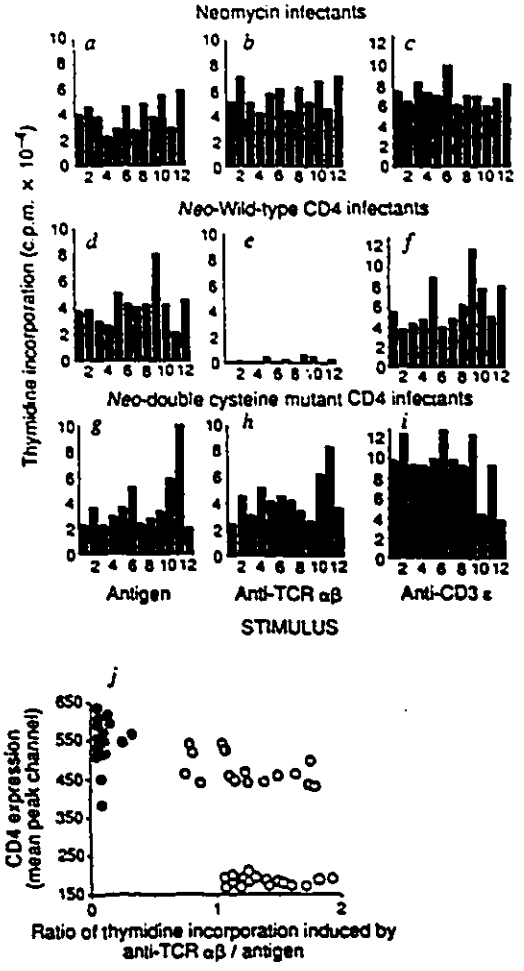

FIG. 2 Association of CD4 with p56^{lck} correlates with lack of response to TCR monoclonal antibody.

METHODS. The *neo* (N), *neo*-wild-type CD4 (NC), and *neo*-mutated CD4 (NDC) infectants of 2.10 were assayed for responsiveness to 100 μg ml^{-1} ovalbumin, 1 μg ml^{-1} anti-TCR$\alpha\beta$ and 1 μg ml^{-1} anti-CD3ε, as described in the legend to Table 1. Responses of 12 representative clones from each category of infection are shown: N (*a–c*), NC (*d–f*), NDC (*g–i*). *j*, 48 stable, G418-resistant clones have been analysed in this study: 15 2.10 NC clones (filled circles), 16 2.10 NDC clones (flecked circles) and 17 2.10 N clones (circles), were assayed for responsiveness to anti-TCR$\alpha\beta$ and to antigen. Levels of CD4 expression were assessed by immunofluorescence and FACS analysis (see legend to Fig. 1*a*). The mean peak channel of fluorescence is plotted on the ordinate and the ratio of thymidine incorporation induced by 1 μg ml^{-1} anti-TCR$\alpha\beta$ divided by that induced by 100 μg ml^{-1} ovalbumin is plotted on the abscissa.

and mutated CD4 infectants of clone 2.10 responded robustly to anti-Vβ4 alone. Importantly, the responses of mutated CD4 infectants were not noticeably enhanced in the presence of both anti-Vβ4 and anti-CD4. Thus, coaggregation of CD4 *per se* with TCR-CD3 does not provide supplementary activation signals. In contrast, in clone 2.5 and wild-type CD4 infectants of clone 2.10, where $p56^{lck}$ is associated with CD4, the induction of DNA synthesis required coaggregation of CD4 and TCRαβ (Table 1*b*).

Calcium ion responses induced through TCRαβ in clones 2.5, 2.10 and CD4 infectants of clone 2.10, parallel results obtained in proliferation assays (Fig. 3*A*). Specifically, aggregation of TCRαβ in the $CD4^-$ clone 2.10, and in mutated CD4 infectants of clone 2.10, results in robust mobilization of intracellular Ca^{2+} involving the majority of cells (Fig. 3*A*, *b*, *g*). Responses of mutated CD4 infectants were not enhanced on coaggregation of CD4 and TCRαβ (compare Fig. 3*A*, *g* with *h*). In contrast, anti-Vβ4 stimulation of clone 2.5 and wild-type CD4 infectants of clone 2.10, results in minimal Ca^{2+} mobilization (Fig. 3*A*, *d*, *j*), which is rescued on coaggregation of CD4 and TCRαβ (Fig. 3*A*, *e*, *k*).

The induction of tyrosine phosphorylation is the first prerequisite signal induced through the T-cell antigen receptor, without which the induction of subsequent activation signals, including the mobilization of intracellular calcium, are blocked[2,3,18]. To assess the role of the CD4-$p56^{lck}$ complex in both prohibiting and enhancing[12] this process, we analysed the accumulation of tyrosine phosphorylated proteins after TCRαβ aggregation in clones 2.5, 2.10, and CD4 infectants of clone 2.10. Aggregation of TCRαβ on clone 2.5 (Fig. 3*B*, *a*) and on wild-type CD4 infectants of clone 2.10 (Fig. 3*B*, *b*) resulted in reduced levels of tyrosine phosphorylation relative to those observed in clone 2.10 (Fig. 3*B*, *a*) and mutated CD4 infectants of clone 2.10 (Fig. 3*B*, *b*). Quantitative analysis revealed that the levels of phosphorylation of at least four substrates in wild-type $CD4^+$ clones were 3–15-fold lower after antigen recep-

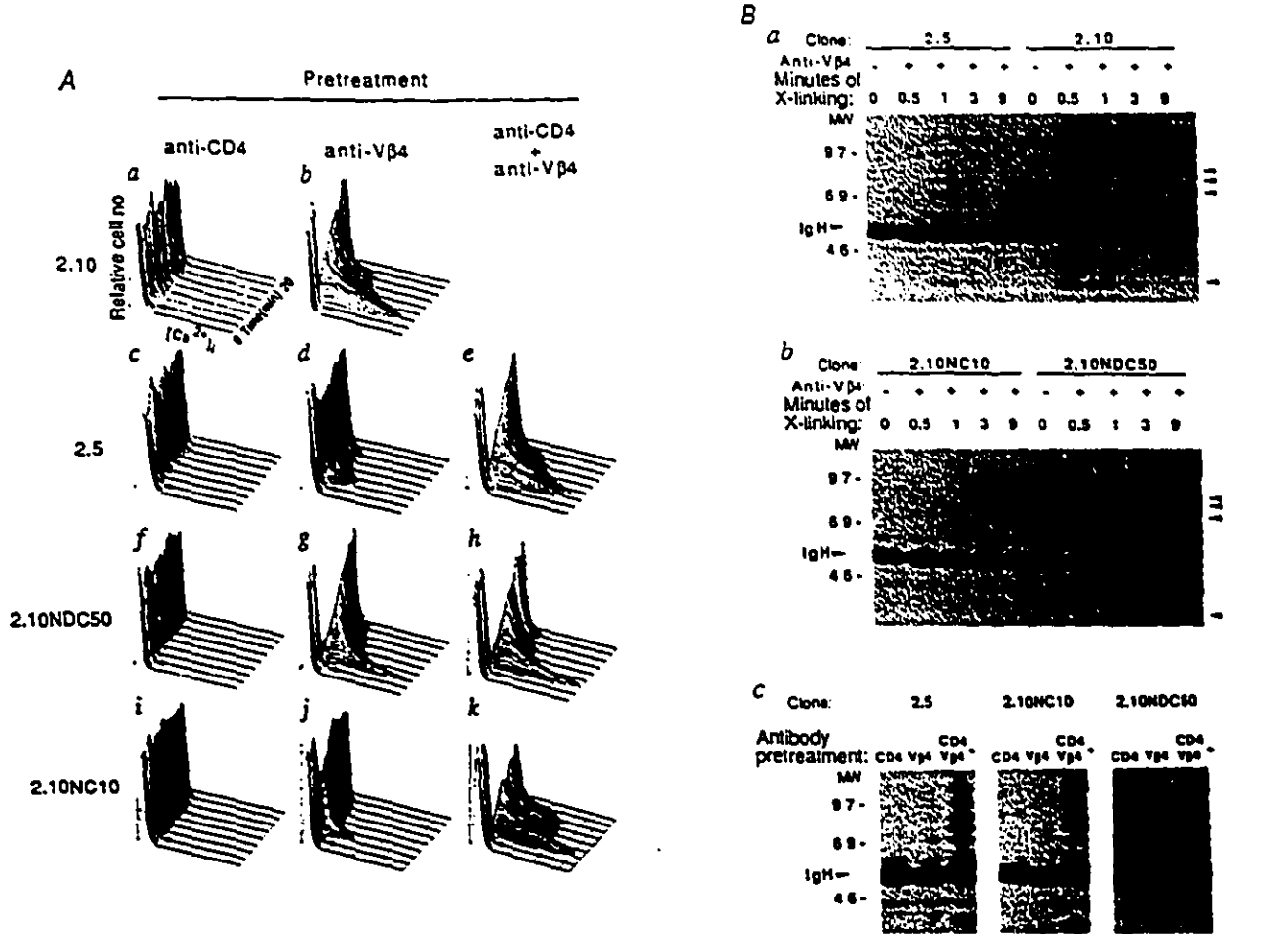

FIG. 3 *A*, Expression of wild-type CD4 inhibits anti-TCRαβ induced calcium mobilization. *B*, Expression of wild-type CD4 inhibits anti-TCRαβ-induced protein tyrosine phosphorylation.

METHODS. *A*, Intracellular calcium concentration [Ca^{2+}], was measured using Indo-1 (Molecular Probes) and a BD FACSTAR plus. Briefly, after loading with Indo-1, cells (at 5×10^6 ml^{-1}) were pretreated with the indicated antibodies specific for CD4 (ref. 37), Vβ4 (ref. 23) or a combination of the two, each at 10 μg ml^{-1}. After washing, cells were passed through the cytometer at a rate of 500 per second to establish a baseline, after which, and indicated by the arrow on each series of histograms, 30 μg ml^{-1} polyclonal mouse anti-rat (Jackson) was added. Histograms were generated using BD Lysis II software, plotting [Ca^{2+}], on the *x* axis, relative cell number on the *y* axis, and time (0–20 min) on the *z* axis. *B*, Anti-phosphotyrosine immunoblotting has been described[4]. Briefly, in panels (*a*) and (*b*), cells at 2.5×10^7 ml^{-1} were pretreated with antibodies specific for Vβ4 (ref. 23), washed, adjusted to 4×10^7 ml^{-1} and brought to 37 °C. Polyclonal mouse anti-rat antiserum was then added to a final concentration of 40 μg ml^{-1}. Crosslinking was done for 0.5, 1, 3 and 9 min after which cells were lysed in boiling sample buffer and fractionated on 10% SDS–PAGE. After transfer to nitrocellulose and blocking, phosphotyrosine-containing proteins were revealed by immunoblotting with anti-P-Y antibody (4G10, ref. 45), followed by ^{125}I-goat anti-mouse IgG (ICN). The zero time point in *a* and *b* represents cells not pretreated with anti-Vβ4, but crosslinked with anti-rat IgG for 1 min. *c*, Cells were pretreated with antibodies specific for CD4, or Vβ4, or a combination of the two before crosslinking for 0.5 min. Fractionation and immunoblotting were done as described above. The relative densities of various molecular species shown on immunoblots in *a* and *b*, indicated by arrows to the right, were quantitated using a phosphorimager (Molecular Dynamics).

tor aggregation for 30 s (Fig. 3*B*, *a* and *b*, indicated by arrows). As shown in Fig. 3*B*, *c*, coaggregation of TCR$\alpha\beta$ and CD4, which is complexed with p56^{lck}, rescues levels of tyrosine phosphorylation comparable to those observed in mutated CD4 infectants of clone 2.10 stimulated with anti-Vβ4 alone.

Thus, the analysis of both early and late antigen receptor-induced signals provides concordant results, which indicate that prerequisite signal(s) are provided by CD4-associated p56^{lck}, and that this complex needs to be close to TCR-CD3. These results can be explained if one considers that in clone 2.5, and in wild-type CD4 infectants of clone 2.10, the majority of cellular p56^{lck} is physically and/or functionally sequestered by CD4 (Fig. 1*b*). This interpretation is consistent with the recent observation that overexpression of wild-type CD4 in mice transgenic for a class I-restricted TCR$\alpha\beta$ inhibited both CD8-dependent proliferative responses, as well as intrathymic positive selection of these class I-restricted T cells[29,30].

In contrast to results presented here, we previously reported that anti-TCR$\alpha\beta$ induces robust DNA synthesis in primary CD4$^+$ T cells[20]. An obvious difference is that although the majority of primary CD4$^+$ T cells are quiescent, the clones used in this study are propagated and maintained in interleukin-2 (IL-2) before assay. Recent reports indicate not only that IL-2 activates cellular p56^{lck} (ref. 31), but also that p56^{lck} may associate with the β-chain of the IL-2 receptor (IL-2r)[32]. Not all cellular p56^{lck} is associated with CD4, ranging from 65 to 95% in primary T cells and clones. It is not implausible that non-CD4-associated p56^{lck} in resting primary T cells is available to interact with antibody-induced aggregates of TCR$\alpha\beta$-CD3, whereas the non-CD4-associated p56^{lck} in clone 2.5 and wild-type CD4 infectants of clone 2.10 may be involved in IL-2r signalling.

Note that previous demonstrations of the functional uncoupling of TCR$\alpha\beta$ and CD3ε in primary mature T cells[20] and thymocytes[33] can now be extended to long-term *in vitro* propagated clones (Table 1*a*; Fig. 2). These results are consistent with the notion that p56^{lck} is required to functionally couple TCR$\alpha\beta$ to the CD3 complex. Further, they imply that p56^{lck} may not be involved in regulating signals generated directly through CD3ε, or alternatively, that its involvement is under different functional and/or physical constraints.

In contrast to previous reports analysing the involvement of CD4 in antigen stimulation[34,35], we have shown that the loss of CD4 by clone 2.10 does not abrogate its response to antigen. Expression of exogenous wild-type CD4 in clone 2.10 reveals a new functional property of this molecule. CD4 prohibits the induction of growth signals through TCR$\alpha\beta$ in the absence of antigen probably by sequestration of p56^{lck}. This in turn provides a molecular mechanism for the functional constraints imposed on T-cell activation by the MHC complex. □

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1. Dianzani, F., Lusher, L. & Dickmann, K. *Eur. J. Immun.* **17**, 529–534 (1987).
2. Owens, T., Fazekas de St Groth, B. & Miller, J. F. A. P. *Proc. natn. Acad. Sci. U.S.A.* **84**, 9209–9213 (1987).
3. Anderson, P., Blue, M.-L., Morimoto, C. & Schlossman, S. F. *J. Immunology* **138**, 678–682 (1987).
4. Abraham, N., Miceli, M. C., Parnes, J. R. & Veillette, A. *Nature* **338**, 62–66 (1991).
5. Veillette, A., Bookman, M. A., Horak, E. M. & Bolen, J. B. *Cell* **55**, 301–308 (1988).
6. Rudd, E. E., Trevillyan, J. M., Dasgupta, J. D., Wong, L. L. & Schlossman, S. F. *Proc. natn. Acad. Sci. U.S.A.* **85**, 5190–5194 (1988).
7. Veillette, A., Bookman, M. A., Horak, E. M., Samelson, L. E. & Bolen, J. B. *Nature* **338**, 257–259 (1989).
8. Kupfer, A., Singer, S. J., Janeway, C. A. Jr & Swain, S. L. *Proc. natn. Acad. Sci. U.S.A.* **84**, 5888–5892 (1987).
9. Doyle, C. & Strominger, J. L. *Nature* **330**, 256–259 (1987).
10. Barber, E. K., Dasgupta, J. D., Schlossman, S. F., Trevillyan, J. M. & Rudd, C. E. *Proc. natn. Acad. Sci. U.S.A.* **86**, 3277–3281 (1989).
11. Chuck, R. S., Clayton, C. R. & Tite, D. B. *Proc. natn. Acad. Sci. U.S.A.* **87**, 5021–5025 (1990).
12. Ledbetter, J. A., Gilliland, L. K. & Schieven, G. L. *Sem. Immun.* **2**, 99–108 (1990).
13. Burgess, K. E. *et al. Eur. J. Immun.* **22**, 1963–1968 (1992).
14. Biddison, W. E., Rao, P. E., Talle, M. A., Goldstein, G. & Shaw, S. *J. exp. Med.* **156**, 1065–1076 (1982).
15. Marrack, P. *et al. J. exp. Med.* **158**, 1077–1091 (1983).
16. Meuer, S. C. *et al. J. Immun.* **130**, 657–664 (1987).
17. Ranoff, P. M., Burakoff, S. J. & Greenstein, J. L. *Cell* **48**, 845–853 (1987).
18. Bierer, L. & Chess, L. *J. exp. Med.* **162**, 1294–1303 (1985).
19. Wassmer, P., Chen, C., Lüdgberg, L. & Shevach, E. M. *J. Immun.* **136**, 2237–2242 (1985).
20. Newell, M. K., Haughn, L. J., Maroun, C. R. & Julius, M. H. *Nature* **347**, 286–289 (1990).
21. Cambier, J. C. & Julius, M. H. *Scand. J. Immun.* **27**, 59–71 (1988).
22. Kubo, R. T. *et al. J. Immun.* **142**, 2736–2742 (1989).
23. Tomonari, K., Lovering, E. & Spencer, S. *Immunogenetics* **31**, 333–339 (1990).
24. Leo, O., Foo, M., Sachs, D. M., Samelson, L. M. & Bluestone, J. A. *Proc. natn. Acad. Sci. U.S.A.* **84**, 1374–1377 (1987).
25. Shaw, A. S. *et al. Cell* **59**, 627–636 (1989).
26. Turner, J. M. *et al. Cell* **60**, 755–765 (1990).
27. Mustelin, T., Coggeshall, K. M., Isakov, N. & Altman, A. *Science* **247**, 1584–1587 (1990).
28. Koretzky, G. A., Picus, J., Thomas, M. L. & Weiss, A. *Nature* **346**, 66–68 (1990).
29. Teh, H.-S. *et al. Nature* **349**, 241–243 (1991).
30. Van Oers, N. S. C. *et al. Eur. J. Immun.* **22**, 735–743 (1992).
31. Horak, I. D. *et al. Proc. natn. Acad. Sci. U.S.A.* **88**, 1996–2000 (1991).
32. Hatakeyama, M. *et al. Science* **252**, 1523–1528 (1991).
33. Finkel, T. H. *et al. J. Immun.* **142**, 3006–3012 (1989).
34. Stedman, B. P. *et al. Nature* **328**, 351–353 (1987).
35. Glaichenhaus, N., Shastri, N., Littman, D. R. & Turner, J. M. *Cell* **64**, 511–520 (1991).
36. Karasuyama, H. & Melchers, F. *Eur. J. Immun.* **18**, 97–104 (1988).
37. Pierres, A. *et al. J. Immun.* **132**, 2775–2783 (1983).
38. Bhattacharya, A., Dorf, M. E. & Springer, T. A. *J. Immun.* **127**, 2488–2495 (1981).
39. Springer, T. A. *Monoclonal Antibodies* 185–192 (Plenum, New York, 1980).
40. Ledbetter, J. & Herzenberg, L. *Immun. Rev.* **47**, 63–90 (1979).
41. Veillette, A. & Fournel, M. *Oncogene* **5**, 1455–1462 (1990).
42. Ho, S. *et al. Gene* **77**, 51–59 (1989).
43. Fleury, S. *et al. Cell* **66**, 1037–1049 (1991).
44. Williams, D. A., Lemischka, I. R., Nathan, D. G. & Mulligan, R. C. *Nature* **310**, 476–480 (1984).
45. Morrison, D. K. *et al. Cell* **58**, 649–657 (1989).

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CHAPTER 3

THE EXTRACELLULAR DOMAIN OF CD4 REGULATES THE INITIATION OF T CELL ACTIVATION

1. Preface

Human T cell clones are very hard to maintain in culture. Transfection of mutated cDNAs to study the function of molecules in T cell activation in human clones is even more laborious. As we were interested in studying the effect of the HIV gp120 on antigenic stimulation by using mutants of CD4, we had to determine if human CD4 behaves like murine CD4 in a murine T cell clone. We thus transfected human CD4 and mutants of human CD4 into the CD4 negative murine T cell clone 2.10. Moreover, transfection of a chimera consisting of the extracellular domain of the epidermal growth factor receptor (EGFR) and the transmembrane and cytoplasmic domains of CD4 allowed us to determine if CD4 can regulate the initiation of T cell activation independently of its interaction with MHC class II molecules.

2. Abstract

We have previously demonstrated that murine CD4 can sequester lck from the TcR and inhibit proliferation induced through the TcR. Stimulation of CD4 positive clones could only be observed if CD4 molecules associated to lck were co-aggregated with the TcR complex, further confirming that the simultaneous interaction of MHC class II molecules with the CD4/lck complex and the TcR is required to initiate T cell activation. To assess the involvement of the extracellular portion of CD4 in the initiation of T cell activation, we transfected a chimeric molecule (EGFRCD4) consisting of the extracellular portion of the epidermal growth factor receptor (EGFR) and of the transmembrane and cytoplasmic domains of human CD4. Our results show that the EGFRCD4 chimera associates with lck and that its oligomerization induced by EGF binding stimulates lck activity. Although the chimera associates with the same levels of lck as wild type CD4 does, chimera expressing clones were very well stimulated to proliferate by anti-TcR Ab in the absence of co-aggregation, demonstrating that the chimera lacks a regulatory domain of CD4. Our results thus suggest that the extracellular portion of CD4 regulates in cis the initiation of T cell activation, most probably by sequestering a molecule also required for the initiation of T cell activation.

3. Introduction

The CD4 molecule is expressed on a subset of T lymphocytes that recognize antigen presented by Major Histocompatibility Complex (MHC) class II molecules. It is comprised of four Immunoglobulin (Ig)-like extracellular domains, a single transmembrane domain and a short cytoplasmic tail of 38 residues. Residues located in the two first Ig-like domains of CD4 interact with non-polymorphic residues on MHC class II molecules (3,5,6,8,18). This interaction is believed to increase the avidity of the TcR for the peptide-MHC complex.

CD4 is associated non-covalently with a src-related tyrosine kinase, $p56^{lck}$ (23,32). This interaction requires two cysteine residues in the cytoplasmic domain of CD4 and two cysteine residues located in the N-terminal portion of lck, and may involve an ion (24,25,30). Cross-linking of CD4 molecules with anti-CD4 antibodies leads to an increase in tyrosine kinase activity of the CD4-associated lck (16,33).

One of the earliest event following TcR stimulation is an increase in tyrosine phosphorylation of several proteins (13). The use of tyrosine kinase inhibitors demonstrated that these tyrosine phosphorylation events are absolutely required to initiate T cell activation (14,26). Several studies have shown that lck plays a crucial role in the initiation of T cell activation. Transfection of an activated form of lck (F505) in a mouse hybridoma enhanced anti-TcR induced IL-2 production (1). Moreover, two lck negative T cell lines were demonstrated to be refractory to anti-TcR stimulation, both at the level of second messenger generation such as calcium influx and tyrosine phosphorylation and later events of T cell activation such as CD69 expression, IL-2 production and cytotoxicity (15,27). Transfection of lck in these cells reconstituted their response tc anti-TcR stimulation demonstrating that their defect was specifically due to the absence of lck. Recent studies link the requirement for lck expression to the activation of the tyrosine kinase ZAP-70 which is also required for early T cell activation events. Indeed, lck was shown to phosphorylate the ζ chain on tyrosine residues allowing the association of ZAP with ζ and the activation of its tyrosine kinase activity (10,12,27).

The role of the CD4/lck association in generating signals regulating T cell activation has been widely studied. Earlier studies demonstrated that cross-linking of CD4 independently of the TcR inhibited activation of T cells (22,28). Moreover, this treatment was shown to prime T cells for apoptosis (2,19). In contrast, co-aggregation of CD4 and the TcR leads to an enhancement of T cell activation (21). The importance of the association of lck with CD4 was revealed in studies which showed that reconstitution of a CD4-dependent antigenic response required the association of CD4 with lck (9). In

addition, we have demonstrated that co-aggregation of the CD4/lck complex with the TcR is required to initiate T cell activation (11). More recent studies have suggested that CD4 also enhances T cell activation independently of its association with lck and its interaction with MHC class II molecules (34,35).

In this report, we further characterize the regulatory role of CD4 in T cell activation. Using a murine T cell clone, we demonstrate that human CD4 can also sequester lck from the TcR leading to down regulation of proliferation induced by anti-TcR Ab. We also designed a chimeric molecule between the Epidermal Growth factor receptor (EGFR) and CD4 to study the role of the extracellular portion of CD4. Our results show that the EGFRCD4 chimera lacks a regulatory function that CD4 possesses which is mapped to the extracellular domain of CD4.

4. Material and methods

Cells

2.10 and its transfected derivatives were grown in IMDM base supplemented with lecithin and recombinant mouse IL-2, as previously described (11).

DNA constructs

Wild type CD4 cDNA and all mutant cDNAs were cloned into the eukaryotic expression vector MNC stuffer coding for the neomycin resistance gene as previously described (29). The generation of the double cysteine mutant was previously described (29). The EGFRCD4 chimera was generated by PCR using the overlap-extension method as described (29). Briefly, the external domain of the EGFR was amplified using a 5' primer complementary to vector sequences and a hybrid 3' primer hybridizing to the last 30 nucleotides of the extracellular portion of the EGFR fused to the first 30 nucleotides coding for the transmembrane domain of CD4. The transmembrane and the cytoplasmic domain of CD4 were amplified using a 3' primer complementary to vector sequences and a 5' primer complementary to the first 30 nucleotides of the CD4 transmembrane domain fused to last 30 nucleotides of the extracellular domain of the EGFR. The full cDNA coding for the chimera was amplified by mixing the two amplified hybrid cDNAs and the 5' and 3' vector primers. The full cDNA was digested with XhoI NotI and was subcloned into the MNC EGFR plasmid also digested with XhoI NotI. The fragment generated by PCR was sequenced using Sanger's method.

Transfections

2.10 cells were transfected by retroviral infection using the DAMP packaging cell line as previously described (29). Transfectants were selected in G418 at 1 mg/ml in either T75 flasks or in a 24 well plate. High expressers of transfected cDNAs were enriched by sorting using the FACSTAR (Becton Dickinson, California). Transfected populations were also cloned at 1 and 5 cells per well.

Functional assays

$5X10^4$ T cells were co-cultured with $5X10^5$ irradiated (2500 rads) freshly isolated splenocytes from C57BL/6 mice , in the presence of either ovalbumin at 200 µg/ml, anti-Vβ4 mAb KT4.10 or anti-CD3 mAb 145-2C11 at 1 µg/ml, or media for about 40 hours at 37°C. The cells were then pulsed with 1 µcurie of [^{3}H]-thymidine (Dupont) for 6 hours. Thymidine incorporation was quantified using the β-plate counter (Pharmacia).

Immunoprecipitations and kinase assays

5-10X10^6 cells were lysed in NP-40 lysis buffer as previously described (29). CD4 was immunoprecipitated using anti-CD4 B66 mAb (4) and the EGFRCD4 chimera was immunoprecipitated with the anti-EGFR mAb 108 (kindly provided by J. Schlessinger) coated on protein A sepharose beads. The immunoprecipitates were washed extensively and proteins were resolved on a 10 % SDS-page gel. For the kinase assays, the immunoprecipitates were incubated with [^{32}P]γ–ATP (Amersham) in kinase buffer for 10 min at room temperature as previously described (29). The kinase reaction was stopped with sample buffer. Proteins were resolved on a 10% SDS-PAGE gel. Bands were quantified by phosphoimager (Molecular Dynamics).

Stimulations for biochemical analysis

For EGF stimulation, cells were incubated with 100 nM EGF (UBI) at 37°C for the indicated time. Stimulated cells were lysed in NP-40 lysis buffer to stop the stimulation. Lysates were treated as described above. For anti-TcR stimulations, cells were incubated with either media or anti-Vβ4 Ab at 10 μg/ml on ice for 30 min. Cells were then washed and resuspended in pre-heated Goat anti-Rat Ab (Jackson Immunoresearch) at 40 μg/ml and incubated at 37°C for the indicated time. For co-crosslinking, cells were incubated with biotinylated anti-Vβ4 Ab at 10 μg/ml and CD4 specific 1F3 mAb at 5 μg/ml on ice for 30 min. Cells were then washed and incubated for 30 min on ice with a biotinylated Goat anti-mouse Ab at 1.5 μg/ml (BRL). Cells were again washed and resuspended in avidin (Sigma) at 50 μg/ml pre-heated at 37°C. The mixture was incubated at 37°C for the indicated time. Stimulation was stopped by lysis of the cells in sample buffer. Samples were then run on a 10% SDS-Page gel.

Western blots

After migration on SDS Page, proteins were transferred onto nitrocellulose. A rabbit anti-lck serum followed by [^{125}I]-protein A, were used to reveal the lck co-immunoprecipitated with either CD4 or the EGFRCD4 chimera. The anti-phosphotyrosine mAb (UBI) followed by [^{125}I] labeled goat anti-mouse Ab were used to reveal tyrosine phosphorylated bands.

5. Results

Human CD4 can sequester lck from the TCR leading to a down regulation of anti-TcR induced proliferation

To verify that human CD4 has the same regulatory properties as murine CD4 when expressed in a murine T cell clone, we transfected the CD4 negative 2.10 T cell clone with either wild type human CD4 or with a mutant of CD4 that has lost the ability to associate with lck , the double cysteine mutant (C420-2A) (figure 1A). As expected, murine lck associates with wild type human CD4 and not with the double cysteine mutant (figure 2C). Also, cross-linking of wild type human CD4 with anti-CD4 monoclonal antibody (mAb) leads to an increase in tyrosine kinase activity of the CD4-associated lck (data not shown). Thus, human CD4 expressed in a murine system seems to have the same biochemical interactions with lck as murine CD4 does.

In confirmation of previous results, this T cell clone is equally induced to proliferate following antigenic stimulation whether it expresses CD4 or not (figure 1B). However, only CD4 negative and double cysteine clones proliferated in response to anti-Vβ4 stimulation. Indeed, cells expressing either murine CD4 (L3T4) or human CD4 were not induced to proliferate by anti-TcR stimulation. The lack of stimulation of wild type human CD4 expressing clones was also observed at the level of tyrosine phosphorylation of substrates (figure 1C). Stimulation of CD4 positive clones could only be observed if the CD4-lck association was co-cross-linked with the TcR (figure 1C). These results confirm that co-aggregation of the CD4/lck complex and the TcR is required to initiate T cell activation.

Design of the EGFRCD4 chimera

To investigate the role of the extracellular portion of CD4 in regulating T cell activation, we designed a chimeric molecule between the EGFR and CD4. The EGFR was chosen because it is not normally expressed in T cells and thus should not associate or interact with other molecules present at the cell surface of the T cell. Moreover, EGF induces oligomerization of the EGFR, thereby stimulating its intrinsic tyrosine kinase

activity (31). This is reminiscent of how the CD4-associated lck tyrosine kinase activity is stimulated using CD4 specific antibodies.

The chimera consists of the extracellular domain of the EGFR, and the transmembrane and cytoplasmic domains of human CD4 (figure 2A). The chimera was transfected in the CD4 negative 2.10 clone and its cell surface expression was detected using an EGFR specific mAb (Figure 2B). The presence of the cytoplasmic domain of CD4 in the EGFRCD4 chimera should confer the ability to associate with lck. To determine this, we immunoprecipitated the chimera with an EGFR specific mAb, and revealed the presence of lck in the immunoprecipitates by a lck specific western blot. As shown in figure 2C, similar amounts of lck are associated with the EGFRCD4 chimera and human CD4. Furthermore, immunodepletion experiments have demonstrated that the same percentage of lck is associated with the chimera as with wild type CD4 (data not shown).

Binding studies have revealed that EGF binds with high affinity to the EGFRCD4 chimera (data not shown). We thus used EGF to oligomerize the chimera and induce lck tyrosine kinase activity. Cells were incubated at 37°C in either media alone or in the presence of EGF at saturating concentrations for an increasing amount of time. Stimulated cells were then lysed and the chimera was immunoprecipitated with an EGFR specific mAb. An autophosphorylation assay was then performed on the immunoprecipitates. As shown in figure 2D, EGF binding to the chimera leads to an increase in chimera-associated lck autophosphorylation activity. Quantification revealed that the increase in lck activity (2-3 fold) is in the range of what is observed following CD4 cross-linking by Abs (data not shown).

In summary, we have designed a chimeric molecule consisting of the extracellular portion of the EGFR, and the transmembrane and cytoplasmic portions of human CD4. This chimera is associated with the same level of lck as wild type CD4 does and upon oligomerization induced by EGF, lck autophosphorylation activity is increased. Thus, the chimera seems to have the same biochemical properties as wild type CD4 regarding its functional association with lck.

The EGFRCD4 chimera does not down regulate anti-TcR induced proliferation

As the EGFRCD4 chimera is associated with the same amount of lck as wild type CD4, we thus expected the chimera to down regulate the induction by anti-TcR Ab of T cell proliferation . Surprisingly, chimera expressing clones were stimulated to proliferate

using either antigen or anti-TcR antibody as stimulators (figure 3A). The ratio of TcR/Ag stimulation was around one as is observed in CD4 negative or double cysteine expressing clones. Tyrosine phosphorylation of substrates was also induced in chimera expressing clones following cross-linking of the TcR using anti-TcR mAb (figure 3B). The pattern, intensity and duration of tyrosine phosphorylation of substrates in chimera expressing clones were similar to those observed in CD4 negative clones. To eliminate variations, we tested clones derived from several independent transfections and always had the same observations (figure 3A and not shown). These results suggest that the EGFRCD4 chimera lacks a regulatory function of CD4 that is mapped to its extracellular domain.

6. Discussion

The response to antigenic stimulation of the T cell clone used in our studies is not enhanced by the presence of CD4. We took advantage of this observation to determine whether the extracellular domain of CD4 regulates the initiation of T cell activation independently of its interaction with MHC class II molecules. This was achieved by the expression of a chimeric molecule which contains the extracellular domain of a cell surface molecule which is not normally expressed in T cells (the EGF receptor), and the transmembrane and cytoplasmic portions of human CD4.

We have previously demonstrated that the association of lck with CD4 requires the presence of an alpha helical structure in the membrane proximal portion of the cytoplasmic tail of CD4 (S. Gratton, submitted). In this report, we show that the extracellular domain of CD4 is not involved in the generation of a proper conformation of its cytoplasmic domain for association with lck. Indeed, a similar amount of lck could be co-precipitated with CD4 and the EGFRCD4 chimera. Binding of EGF to its receptor induces its oligomerization, leading to activation of its intrinsic tyrosine kinase by transphosphorylation (31). Our results show that activation of the chimera-associated lck can be induced by binding of EGF. This suggests that the activation of the CD4-associated lck is not due to a specific change in conformation of CD4 but rather to an oligomerization of CD4. These results thus support the hypothesis that like growth factor receptor tyrosine kinase activity, lck activity can be activated by transphosphorylation.

We have previously demonstrated that murine CD4 can sequester lck and inhibit anti-TcR induced proliferation (11). Co-aggregation of the CD4/lck complex with the TcR normally mediated by MHC class II molecules is required to initiate T cell activation. In this report, we show that the extracellular domain of CD4 regulates the initiation of T cell activation independently of its interaction with MHC class II molecules. Indeed, although the EGFRCD4 chimera sequesters a similar amount of lck as wild type CD4, clones expressing the chimera were induced to proliferate with anti-TcR antibody without being co-aggregated with the TcR. In fact, clones expressing the EGFRCD4 chimera behave like CD4 negative clones and clones expressing the double cysteine mutant, which is not associated with lck. These results suggest that the EGFRCD4 chimera lacks a regulatory function of wild type CD4 that is mapped to its extracellular domain.

How does the extracellular domain regulates T cell activation? It was recently proposed that CD4 and the TcR can interact together and enhance the avidity of the TcR for the peptide-MHC complex (34,35). However, we feel that our observations cannot be

explained by this hypothesis because this clone does not require the expression of CD4 to respond to antigen and its response to antigen is not augmented by the presence of CD4. Moreover, whereas in CD4 expressing clones the down regulation of anti-TcR induced proliferation is due to a lack of co-aggregation of CD4 and the TcR, chimera expressing clones are well stimulated with anti-TcR Ab thus eliminating the hypothesis that the regulatory function of CD4 is association with the TcR.

The hypothesis we favor is that the extracellular domain of CD4 mediates its association with another molecule which is implicated in early T cell activation events. Sequestration of both this molecule and lck would be required to observe down regulation of anti-TcR induced proliferation. Indeed, wild type CD4 would sequester both lck and this other molecule, leading to inhibition of stimulation through the TcR. In contrast, the EGFRCD4 which lacks the CD4 extracellular domain would sequester only lck and thus allow TcR-induced proliferation. Similarly, the mutant of CD4 which does not associate with lck still expresses the extracellular domain of CD4 and thus permits anti-TcR induced stimulation.

An interesting candidate for this association would be CD45. Association of CD45 with CD4 was previously described to be isoform specific (7,17). Since CD45 isoforms are defined by the differential use of extracellular coding exons, this supports the hypothesis of an extracellular mediated interaction between these two molecules. Moreover, it was recently demonstrated that reconstitution of a CD4-dependent response of a T cell clone varied with the isoform of CD45 expressed (20). Isoforms of CD45 which associate with CD4 led to higher responses to antigenic stimulation than other isoforms. Thus, CD4 would potentiate the response of T cells through its association with CD45. This would ensure that a proper amount of tyrosine phosphatase is the proximity of the tyrosine kinase fyn and lck to activate them and induce phosphorylation of the CD3 and ζ chains.

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7. References

1. Abraham, N., M. C. Miceli, J. R. Parnes, and A. Veillette. 1991. Enhancement of T-cell responsiveness by the lymphocyte-specific tyrosine protein kinase $p56^{lck}$. Nature 350:62-66.

2. Banda, N. K., J. Bernier, D. K. Kurahara, R. Kurrle, W. Haigwood, R. P. Sekaly, and T. Finkel. 1992. Crosslinking CD4 by human immunodeficiency virus gp120 primes T cells for activation-induced apoptosis. J. Exp. Med. 176:1099-1106.

3. Bowman, M. R., K. D. MacFerrin, S. L. Schreiber, and S. J. Burakoff. 1990. Identification and structural analysis of residues in the V1 region of CD4 involved in interaction with human immunodeficiency virus envelope glycoprotein gp120 and class II major histocompatibility complex molecules. Proc. Natl. Acad. Sci. USA 87:9052-9056.

4. Carrel, S., A. Moretta, G. Pantaleo, G. Tambussi, P. Isler, B. Perussia, and J. C. Cerottini. 1988. Stimulation and proliferation of CD4+ peripheral blood T lymphocytes induced by an anti-CD4 monoclonal antibody. Eur. J. Immunol. 18:333-339.

5. Clayton, L. K., R. E. Hussey, R. Steinbrich, H. Ramachandran, Y. Husain, and E. L. Reinherz. 1988. Substitution of murine for human CD4 residues identifies amino acids critical for HIV-gp120 binding. Nature 335:363-366.

6. Clayton, L. K., M. Sieh, D. A. Pious, and E. L. Reinherz. 1989. Identification of human CD4 residues affecting class II MHC versus HIV-1 gp120 binding. Nature 339:548-551.

7. Dianzani, U., U. Redoglia, F. Malavasi, M. Bragardo, A. Pileri, C. A. Janeway, and K. Bottomly. 1992. Isoform-specific associations of CD45 with accessory molecules in human T lymphocytes. Eur. J. Immunol. 22:365-371.

8. Fleury, S., D. Lamarre, S. Meloche, S.-E. Ryu, C. Cantin, W. A. Hendrickson, and R.-P. Sekaly. 1991. Mutational analysis of the interaction between CD4 and class II MHC: class II antigens contact CD4 on a surface opposite to the gp120-binding site. Cell 66:1037-1049.

9. Glaichenhaus, N., N. Shastri, D. R. Littman, and J. M. Turner. 1991. Requirement for association of $p56^{lck}$ with CD4 in antigen-specific signal transduction in T cells. Cell 64:511-520.

10. Hall, C. G., J. Sancho, and C. Terhorst. 1993. Reconstitution of T cell receptor z-mediated calcium mobilization in non lymphoid cells. Science 261:915-917.

11. Haughn, L., S. Gratton, L. Caron, R. P. Sekaly, A. Veillette, and M. Julius. 1992. Association of tyrosine kinase $p56^{lck}$ with CD4 inhibits the induction of growth through the ab T - cell receptor. Nature 358:328-331.

12. Iwashima, M., B. A. Irving, N. S. van Oers, A. C. Chan, and A. Weiss. 1994. Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. Science 263:1136-1139.

13. June, C. H., M. C. Fletcher, J. A. Ledbetter, and L. E. Samelson. 1990. Increases in tyrosine phosphorylation are detectable before phospholipase C activation after T cell receptor stimulation. J. Immunol. 144:1591-1599.

14. June, C. H., M. C. Fletcher, J. A. Ledbetter, G. L. Schieven, J. N. Siegel, A. F. Phillips, and L. E. Samelson. 1990. Inhibition of tyrosine phosphorylation prevents T cell receptor mediated signal transduction. Proc. Natl. Acad. Sci. USA 87:7722-7726.

15. Karnitz, L., S. L. Sutor, T. Torigoe, J. C. Reed, M. P. Bell, D. J. McKean, P. J. Leibson, and R. T. Abraham. 1992. Effects of $p56^{lck}$ deficiency on the growth and cytolytic effector function of an interleukin-2-dependent cytotoxic T-cell line. Mol. Cell. Biol. 12:4521-4530.

16. Luo, K. and B. M. Sefton. 1990. Cross-linking of T-cell surface molecules CD4 and CD8 stimulates phosphorylation of the lck tyrosine protein kinase at the autophosphorylation site. Mol. Cell. Biol. 10:5305-5313.

17. Mittler, R. S., B. M. Rankin, and P. A. Kiener. 1991. Physical associations between CD45 and CD4 or CD8 occur as late activation events in antigen receptor-stimulated human T cells. Journal. of. Immunology 147:3434-3440.

18. Moebius, U., P. Pallai, S. C. Harrison, and E. L. Reinherz. 1993. Delineation of an extended surface contact area on human CD4 involved in class II major complex binding. Proc. Natl. Acad. Sci. USA 90:8259-8263.

19. Newell, M. K., L. J. Haughn, C. R. Maroun, and M. H. Julius. 1990. Death of mature T cells by separate ligation of CD4 and the T-cell receptor for antigen. Nature 347:286-289.

20. Novak, T. J., D. Farber, D. Leitenberg, S. C. Hong, P. Johnson, and K. Bottomly. 1994. Isoforms of the transmembrane tyrosine phosphatase CD45 differentially affect T cell recognition. Immunity. 1:109-119.

21. Owens, T., B. Fazekas de St-Groth, and J. F. A. P. Miller. 1987. Coaggregation of the T-cell receptor with CD4 and other T-cell surface molecules enhances T-cell activation. Proc. Natl. Acad. Sci. USA 84:9209

22. Rosoff, P. M., S. J. Burakoff, and J. L. Greenstein. 1987. The role of L3T4 molecule in mitogen and antigen activated signal transduction. Cell 49:845-853.

23. Rudd, C. E., J. M. Trevillyan, J. V. Dasgupta, L. L. Wong, and S. F. Schlossman. 1988. The CD4 antigen is complexed in detergent lysates with the protein tyrosine kinase (pp58) from human T lymphocytes. Proc. Natl. Acad. Sci. USA 85:5190

24. Shaw, A. S., K. E. Amrein, C. Hammond, D. F. Stern, B. M. Sefton, and J. K. Rose. 1989. The cytoplasmic domain of CD4 interacts with the tyrosine protein kinase, $p56^{lck}$, through its unique amino-terminal domain. Cell 59:627

25. Shaw, A. S., J. Chalupny, A. Whitney, C. Hammond, K. E. Amrein, P. Kavathas, B. M. Sefton, and J. K. Rose. 1990. Short related sequences in the cytoplasmic domains of CD4 and CD8 mediated binding to the amino-terminal domain of the $p56^{lck}$ tyrosine protein kinase. Mol. Cell. Biol. 10:1853-1862.

26. Stanley, J. B., R. Gorczynski, C. Huang, J. Love, and G. B. Mills. 1990. Tyrosine phosphorylation is an obligatory event in Il-2 secretion. J. Immunol. 145:2189-2198.

27. Strauss, B. and A. Weiss. 1992. Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor. Cell 70:585-593.

28. Tite, J. P., A. Sloan, and C. A. Janeway. 1986. The role of L3T4 in T-cell activation: L3T4 may be both an Ia binding protein and receptor that transduces a negative signal. J. Mol. Cell. Immunol. 2:179

29. Tremblay, M., S. Meloche, S. Gratton, M. A. Wainberg, and R. P. Sekaly. 1994. Association of $p56^{lck}$ with the cytoplasmic domain of CD4 modulates HIV-1 expression. EMBO J. 13:774-783.

30. Turner, J. M., M. H. Brodsky, B. A. Irving, S. D. Levin, R. M. Perlmutter, and D. R. Littman. 1990. Interaction of the unique N-terminal region of tyrosine kinase $p56^{lck}$ with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. Cell 60:755-765.

31. Ullrich, A. and J. Schlessinger. 1990. Signal transduction by receptors with tyrosine kinase activity. Cell 61:203-212.

32. Veillette, A., M. A. Bookman, E. M. Horak, and J. B. Bolen. 1988. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase $p56^{lck}$. Cell 55:301-308.

33. Veillette, A., M. A. Bookman, E. M. Horak, L. E. Samelson, and J. B. Bolen. 1989. Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase $p56^{lck}$. Nature 338:257

34. Vignali, D. A. and J. L. Strominger. 1994. Amino acid residues that flank core peptide epitopes and the extracellular domains of CD4 modulate differential signaling through the T cell receptor. Journal of Experimental Medicine 179:1945-1956.

35. Zerbib, A. C., A. B. Reske-Kunz, P. Lock, and R. P. Sekaly. 1994. CD4-mediated enhancement or inhibition of T cell activation does not require the CD4:$p56^{lck}$ association. Journal. of. Experimental. Medicine 179:1973-1983.

Figure Legends

Figure 1

Human CD4 behaves like murine CD4. A) Cell surface staining of transfectants. Cells were stained with CD4 specific L93 mAb followed by a Phycoerythrin (PE) conjugated Goat anti-Mouse Ab (Southern Biotechnologies Associates) or with only the PE Goat anti-mouse Ab as a negative control. B) Human CD4 sequesters murine lck and downregulates anti-TcR induced proliferation. Cells transfected with the indicated constructs were stimulated with either ovalbumin or anti-Vβ4 Ab as described in the material and methods. Response of representative clones are depicted. C) Activation through the TcR requires co-aggregation of the CD4/lck association with the TcR complex. Lanes 1,2,3 are lysates from CD4 negative cells and lanes 4,5,6 are lysates from CD4 positive T cells. Cells were stimulated as described in material and methods for 1 min at 37°C with avidin only (lane 1, 4), biotinylated anti-TcR Ab cross-linked by avidin (lane 2,5), or with a combination of anti-CD4 mAb, biotinylated Goat anti-Mouse Ab and biotinylated anti-TcR Ab, cross-linked by avidin (lane 3,6).

Figure 2

Characteristics of the EGFRCD4 chimera. A) Schematic representation of the domains of the EGFRCD4 chimera. B) Cell surface staining of the EGFRCD4 chimera. Cells were stained using the EGFR specific 108 mAb followed by PE Goat anti-mouse Ab (Southern Biotechnologies Associates), or only with the PE Goat anti-mouse Ab as a negative control. C) The EGFRCD4 chimera is associated with lck. CD4 was immunoprecipitated from lysates coming from CD4 negative cells (lane 1), wild type CD4 expressing cells (lane 2,3) and mutant C420-2A expressing cells (lane 4). Lanes 5,6,7 are anti-EGFR immunoprecipitations performed on lysates coming from EGFRCD4 negative cells (lane 5) or from EGFRCD4 positive cells (lane 6,7). The presence of lck in the immunoprecipitates was revealed by hybridization with an anti-lck serum followed by [^{125}I]-labeled protein A. D) EGF stimulates the autophosphorylation activity of the EGFRCD4 associated lck. Cells were stimulated with 100 nM EGF for the indicated times at 37°C. Stimulations were stopped by lysis in NP-40 buffer. The EGFRCD4 chimera was immunoprecipitated with an EGFR specific mAb and a kinase assay was performed on the immunoprecipitates as described in material and methods. Proteins were resolved on a 10% SDS Page gel.

Figure 3

The EGFRCD4 chimera does not down regulate anti-TcR stimulation. A) Cells were stimulated with either ovalbumin or anti-Vβ4 Ab as described in material and methods. Shown are the responses of three representative clones expressing the EGFRCD4 chimera. B) Cells transfected with the MNC vector, CD4 or EGFRCD4 were stimulated with anti-Vβ4 Ab crosslinked with Rabbit anti-Rat Ab at 37°C for the indicated times. Stimulations were stopped by sample buffer. Proteins were resolved on a 10% SDS-Page gel. The presence of tyrosine phosphorylated proteins was revealed by hybridization with an anti-phosphotyrosine mAb (UBI), followed by [^{125}I]-labeled Goat-anti-mouse.

FIGURE 1A

A)

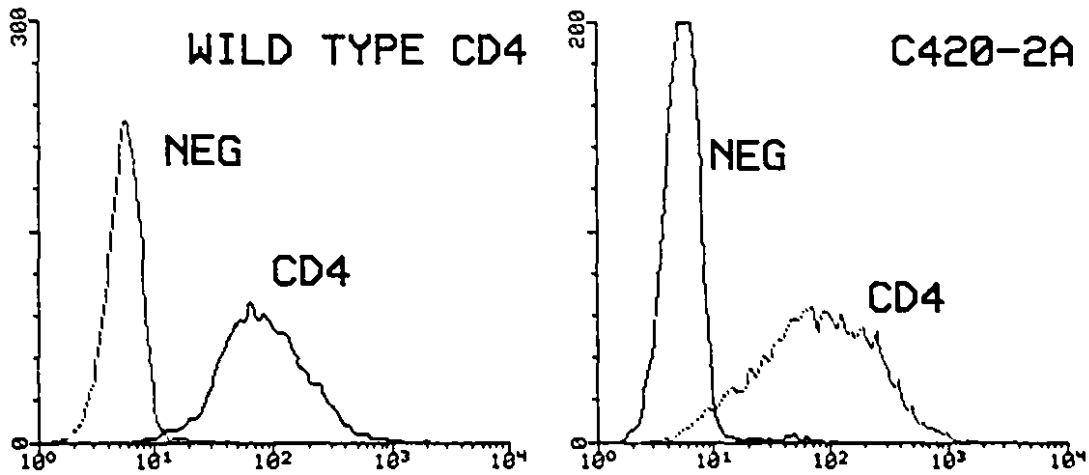


FIGURE 1B

B)

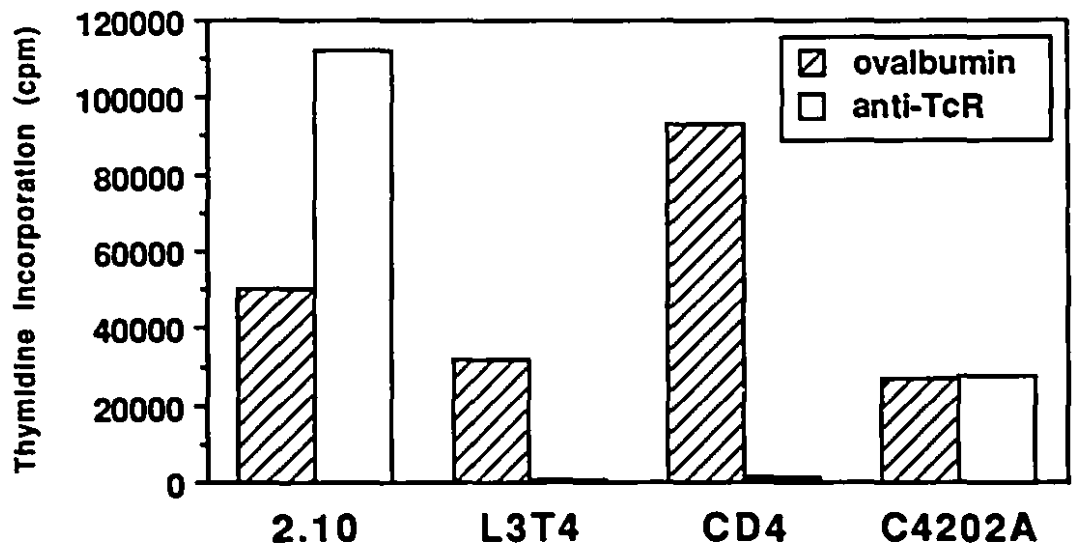

Thymidine Incorporation (cpm)
120000
100000
80000
60000
40000
20000
0
ovalbumin
anti-TcR
2.10
L3T4
CD4
C4202A

FIGURE 1C

C)

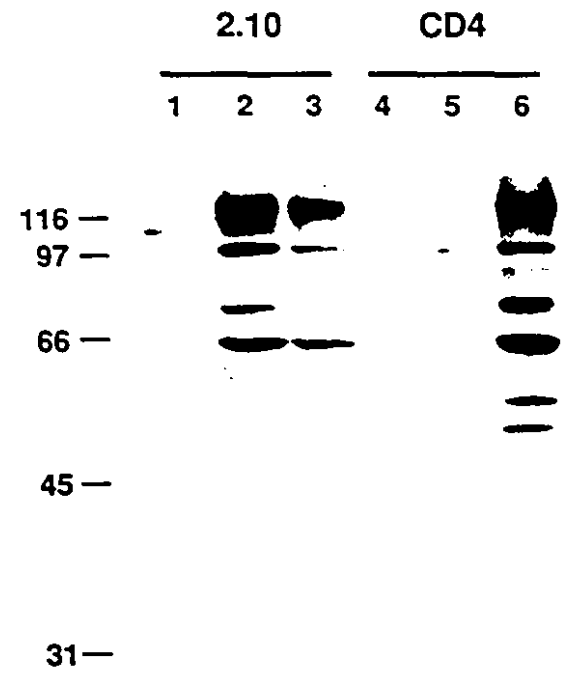
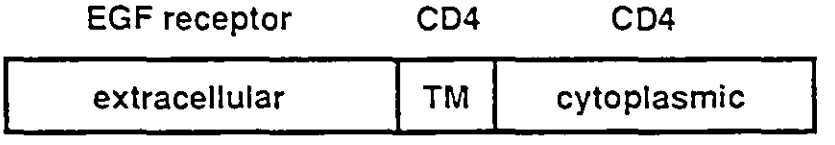


FIGURE 2A and 2B

A)



B)

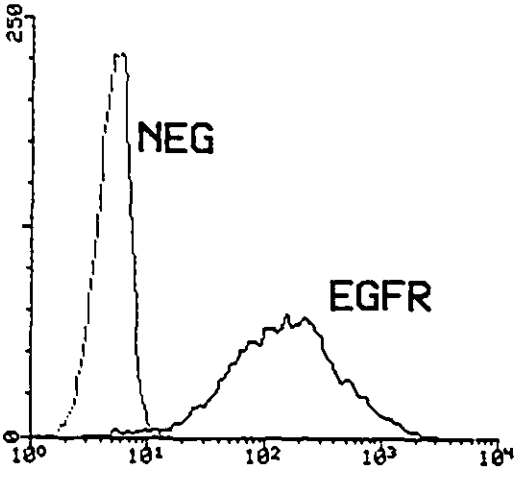


FIGURE 2C and D

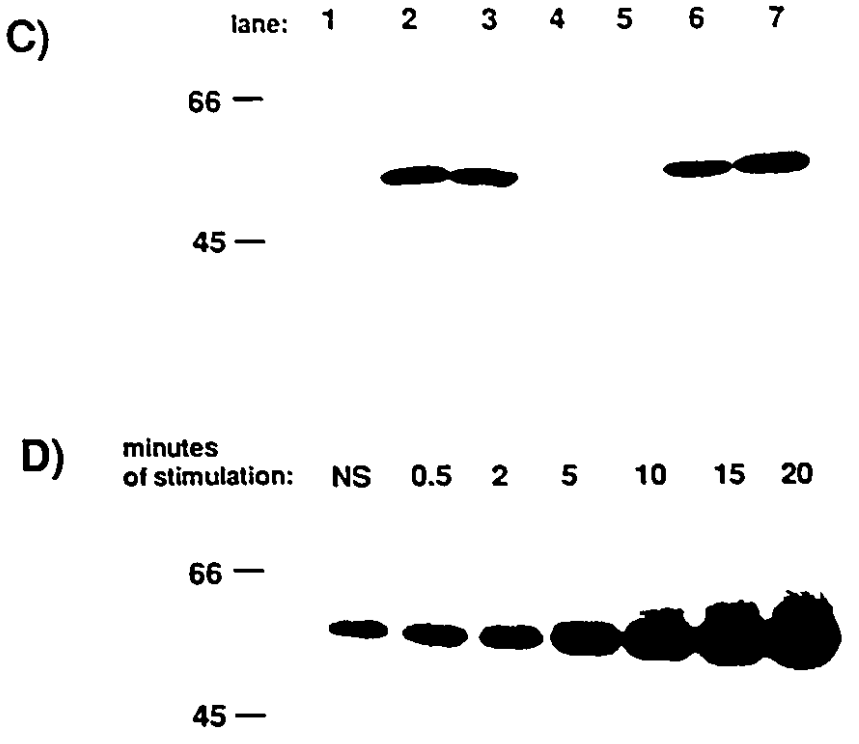


FIGURE 3A

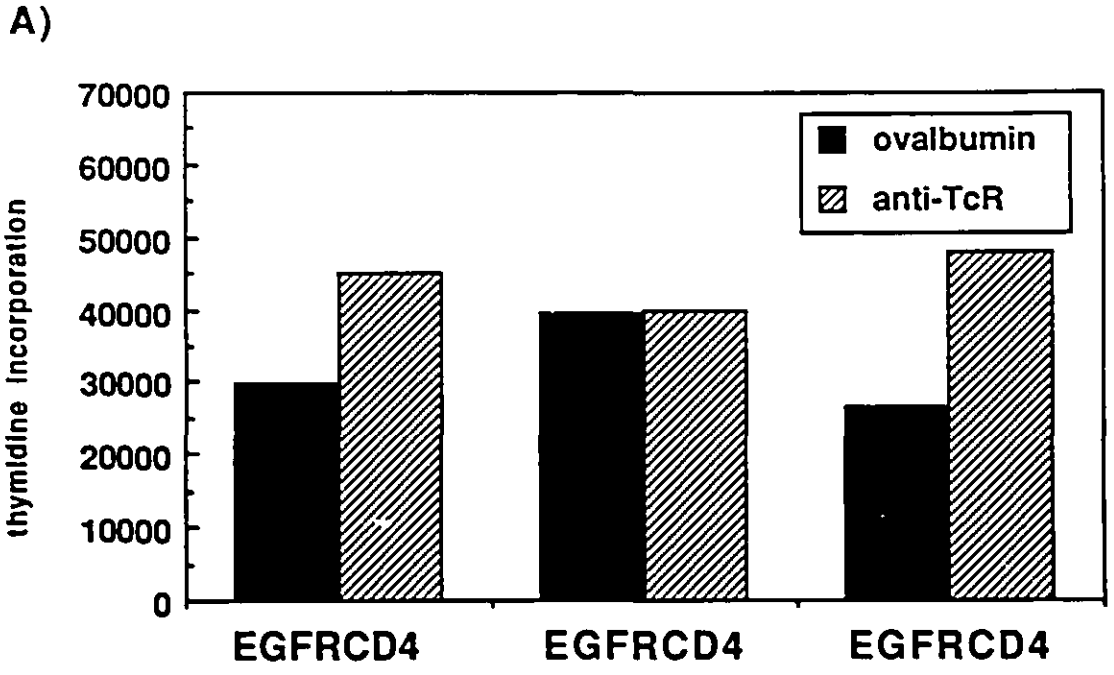
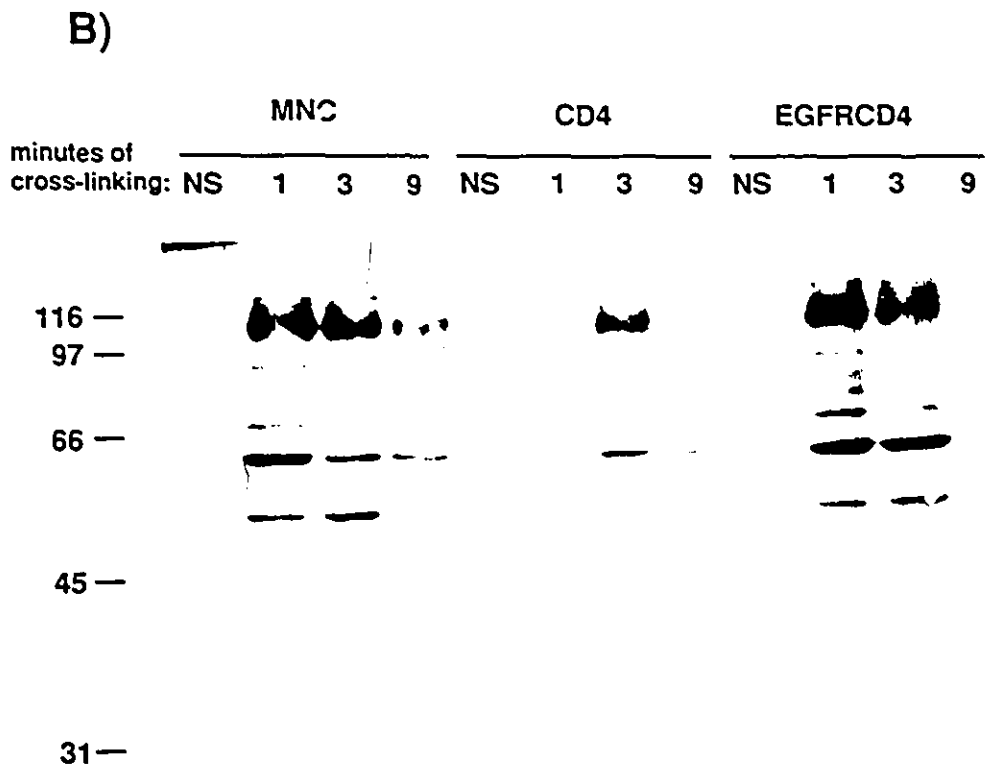

A)
70000
60000
50000
40000
30000
20000
10000
0
thymidine incorporation
ovalbumin
anti-TcR
EGFRCD4
EGFRCD4
EGFRCD4

FIGURE 3B


B)
MNC
CD4
EGFRCD4
minutes of
cross-linking:
NS 1 3 9 NS 1 3 9 NS 1 3 9
116
97
66
45
31

CHAPTER 4

lck independent inhibition of antigenic stimulation by the HIV gp120 glycoprotein

1. Preface

AIDS is characterized by a severe immunodeficiency. T cells isolated from HIV infected individuals have a decreased response to stimulation. Treatment of CD4 T cells with gp120 was previously shown to inhibit T cell activation. We used the 2.10 murine T cell clone transfected with human CD4 to study the mechanism of the inhibitory effect of gp120. Using mutants of CD4, we also defined the requirement for the CD4/lck association in this inhibition.

2. Abstract

Binding of the HIV envelope glycoprotein gp120 to CD4 inhibits T cell activation. We used a murine T cell clone to characterize the pathways involved in the inhibitory effect of gp120 on antigen-induced T cell activation. Antigen-induced proliferation of T cell clones transfected with human CD4 was substantially inhibited in the presence of gp120, even though activation does not require *per se* the CD4/MHC class II interaction. This inhibition by gp120 was not dependent on the association of lck with CD4. Conversely, stimulation of clones expressing a chimera between the EGFR and CD4 (which is functionally associated with lck) was not inhibited by EGF. These results demonstrate that the inhibition by gp120 is not due to the sequestration of lck from TcR and does not require activation of lck by gp120. They rather suggest that CD4 can regulate the initiation of T cell activation independently of the interaction with lck or with class II molecules. Finally, we demonstrate that this non-responsiveness induced by gp120 can be totally reversed by soluble CD4 when added early after start of stimulation. The use of synchronized populations suggested that gp120 exerts its inhibitory effect when cells are in the G0\G1 phase of the cell cycle.

3. Introduction

CD4 positive T lymphocytes recognize antigen presented by MHC class II molecules. The interaction between CD4 and MHC class II molecules was first reported to enhance the response of T cells induced by antigenic stimulation. The cytoplasmic domain of CD4 is non-covalently associated with the src-related tyrosine kinase, $p56^{lck}$ (26,36). Two cysteine residues at positions 420 and 422 of CD4 mediate its interaction with the N-terminal portion of lck (28,29,35). Cross-linking of CD4 molecules using CD4 specific antibodies (Ab) leads to an increase in tyrosine kinase activity of the CD4-associated lck (17,37). This increase in tyrosine kinase activity results in tyrosine phosphorylation of the ζ chain of the TcR complex (37).

The expression of lck is absolutely required to initiate T cell activation (14,33). The activity of lck is implicated in the phosphorylation of the ζ chain and the subsequent recruitment and activation of the ZAP-70 tyrosine kinase (9,12,33). Early studies demonstrated that association of lck with CD4 is required to reconstitute CD4-dependent antigenic stimulation of T cells (7). We have previously shown that CD4 can sequester lck and thus inhibit anti-TcR induced proliferation (10). In order to have an optimal stimulation, co-aggregation of the CD4/lck complex with the TcR through the simultaneous interaction with the same MHC class II molecule is required (5,10).

Signals generated through the CD4/lck complex can also negatively regulate T cell activation. Indeed, cross-linking of CD4 with antibody (Ab) independently of the TcR/CD3 complex inhibits T cell activation, leading to a state of anergy and/or to priming of T cells for apoptosis (1,21,25,34). This negative signal occurs in a pathogenic situation, i.e. in Human Immunodeficiency (HIV) infection.

The CD4 molecule is the physiological receptor for the HIV. The HIV envelope glycoprotein gp120 binds specifically to CD4 leading to viral entry. gp120 was demonstrated to induce lck activity, tyrosine phosphorylation of substrates, calcium influx and the expression of activation markers such as HLA-DR and the IL-2 receptor (8,13,15,32). However, treatment of CD4 positive human T cells with gp120 inhibits stimulation induced by anti-TcR Ab or specific antigen, leading to the induction of anergy (3,4,18,19,22).

Although a correlation can be drawn between stimulation of lck kinase activity and induction of anergy by gp120, no studies have rigorously inquired about the absolute requirement for an increase in lck activity to induce T cell non-responsiveness following treatment by gp120. To further characterize the inhibition of antigenic stimulation induced

by gp120, we have used a murine T cell clone which does not require CD4 expression to respond to antigenic stimulation. Interestingly, stimulation of clones expressing human CD4 was inhibited in the presence of gp120. This allowed the characterization of a regulatory function associated with the CD4 extracellular domain which is independent of its interaction with MHC class II molecules and its association with lck.

4. Material and Methods

Cell lines and reagents

2.10 and transfected derivatives are described elsewhere (Gratton et al, in preparation). Cells were grown in IMDM supplemented with IL-2 and lecithin as previously described (10). C57BL/6 mice (4-6 weeks old) were purchased from Charles River. Splenocytes were isolated from the mice as previously described (10). Recombinant gp120 BH10 was obtained from A.Truneh of Smith Kline Beechem and recombinant soluble CD4 was obtained from Genentech.

Antigenic stimulation

$5X10^4$ T cells were stimulated with $5X10^5$ C57BL/6 splenocytes irradiated (2500 rads) and ovalbumin (Sigma) at the indicated concentrations. Recombinant gp120 or soluble CD4 were added when mentioned at a final concentration of 5 µg/ml. When mentioned in the text, EGF (UBI) was added at a final concentration of 100 nM. Cells were stimulated for about 40 hours at 37°C and then pulsed for 6 hours with 1µcurie of ^{3}H-Thymidine (Dupont).

Synchronization and Cell cycle Analysis

Cells were synchronized by starving them of IL-2 for 2 hours at 37°C and further culturing them in limiting amount of IL-2 (0.05%) for 12 hours at 37°C. For analysis, cells were stained using a modified Krishan buffer. Briefly, cells were fixed 50% ethanol. Cells were then incubated in modified Krishan buffer (0.1% sodium citrate, 0.02 mg/ml RNase, 0.3% NP-40, 0.05 mg/ml propidium iodide) for 30 min on ice. Cells were then centrifuged and resuspended in fresh Krishan buffer. Samples were analyzed on a FACSTAR plus (Becton Dickinson).

5. Results and Discussion

gp120 inhibits antigenic stimulation of a murine T cell clone transfected with human CD4

To characterize the mechanism of inhibition of antigenic stimulation by gp120, a CD4 negative murine T cell clone, 2.10, which recognizes ovalbumin in the context of I-A^b, was transfected with human CD4. This T cell clone was previously shown to be CD4 independent since it proliferates in response to stimulation by antigen to the same extent whether it expresses CD4 or not (10).

As shown in figure 1, gp120 inhibited over 95% of antigen-induced proliferation of a human CD4 positive 2.10 clone in a dose-dependent fashion. gp120 treatment of CD4 negative cells did not affect the response to antigen (data not shown). Whether the monomeric form or an oligomeric form of gp120 is required to observe inhibition of activation is still controversial. In our hands, the monomeric form of gp120 was sufficient to inhibit antigen-induced stimulation, suggesting that cross-linking of CD4 is not required for this effect. Furthermore, inhibition was observed even at high concentrations of antigen, demonstrating the high potency of gp120 to inhibit antigenic stimulation (figure 1B). These results further support the hypothesis that gp120 contributes *in vivo* to the impaired immune response observed in HIV infected individuals.

Inhibition of antigenic stimulation by gp120 does not require the association of lck with CD4

The inhibitory effect of gp120 cannot be explained by a disruption of the adhesion interaction between CD4 and MHC class II molecules as we have previously shown that the response of this T cell clone is not dependent on this interaction (10).

gp120 was previously demonstrated to increase the CD4-associated lck tyrosine kinase activity (8,13,15,22,32). This increase in lck activity was concomitant in some studies with an influx of calcium, tyrosine phosphorylation of substrates and induction of anergy (4,8,18,19). Furthermore, it was demonstrated that treatment of a human T cell clone with gp120 led to internalization of CD4 (3). To determine if these events induced by gp120 are required to observe inhibition of antigenic stimulation, the CD4 negative 2.10 clone was transfected with mutants of CD4 that have lost the ability to associate with lck or to endocytose.

The first mutant tested was the CD4 3S in which three serine residues at positions 408-15-31 of the cytoplasmic domain of CD4 were mutated to alanines. These serine residues become phosphorylated upon antigenic stimulation or PMA treatment, leading to dissociation of lck from CD4 and internalization and degradation of CD4 (2,11,23,24,30,31). Several clones expressing this mutant were derived. Interestingly, these clones still responded to antigen stimulation (figure 2A) whereas this mutation was previously found not to restore a CD4 dependent response (7). Furthermore, gp120 inhibited antigen-induced proliferation of these transfectants (figure 2A). This result suggests that inhibition of antigenic stimulation by gp120 is not due to endocytosis of CD4 or dissociation of lck from CD4.

To verify if lck association with CD4 is required for inhibition by gp120, several 2.10 clones expressing a mutant of CD4 which does not associate with lck, C4202A, were stimulated in the presence of gp120. A representative experiment is shown in figure 2A. Interestingly, gp120 inhibited the antigen induced proliferation of C4202A clones. This result clearly demonstrates that the inhibition of antigenic stimulation induced by gp120 does not require the association of lck with CD4.

Inhibition of antigenic stimulation by gp120 does not require an increase in lck activity

To further characterize the potential contribution of lck activity in inhibition by gp120, we have transfected the 2.10 clone with a chimeric molecule consisting of the extracellular domain of the epidermal growth factor receptor (EGFR) and the transmembrane and cytoplasmic domains of human CD4. We have previously demonstrated that this chimera is associated with a similar amount of lck as wild type CD4 (Gratton S., in preparation). Moreover, binding of EGF to the chimera activates lck tyrosine kinase activity (Gratton S., in preparation). Several chimera-expressing clones were generated and a representative experiment is shown in figure 2B. These clones were well stimulated to proliferate by antigen. Interestingly, no inhibition of antigenic stimulation could be observed in the presence of EGF at saturating concentrations. These results further demonstrate that lck activation is not required or sufficient to observe gp120 inhibition of stimulation by antigen.

CD4 can sequester lck and inhibit anti-TcR induced proliferation if not co-aggregated with the TcR (10). The inhibition of antigen-induced stimulation by gp120 that

we now observe cannot be explained by such a sequestration of lck since clones expressing a mutant of CD4 which does not associate with lck are also inhibited by gp120.

To our knowledge, this is the first report demonstrating that the inhibition of T cell activation by gp120 does not require neither lck association to CD4 or activation of the lck tyrosine kinase activity. Interestingly, treatment with CD4 specific Ab also inhibited antigenic stimulation of T cells expressing a mutant of CD4 which is not associated with lck (39). Taken together, the results presented here define a functional role of CD4 which is independent of its association with lck and its interaction with MHC class II molecules. Binding of gp120 to the external domain of CD4 would mask this regulatory domain and prevent the initiation of T cell activation. gp120 could act by disrupting CD4 dimers. It was recently reported that gp120 binds to monomeric forms of CD4 whereas MHC class II molecules interact with dimers of CD4 (27).

Alternatively, gp120 could modulate the function of another T cell surface molecule which is associated with CD4 and implicated in early T cell activation events. Indeed, CD4 associates with the tyrosine phosphatase CD45 and the TcR complex (6,16,20,38). It is conceivable that binding of gp120 to CD4 could prevent an extracellular interaction between CD4 and the TcR necessary to stabilize the interaction between the TcR and MHC class II molecules. However, the presence of CD4 does not enhance the response of these T cells suggesting that this model is unlikely. Alternatively, gp120 binding to CD4 could sterically prevent an antigen specific TcR/MHC class II interaction or disrupt a regulatory interaction between CD4 and CD45. This last scenario would lead to modulation of CD45 activity and inhibition of T cell activation.

gp120 prevents the formation of blasts

To further determine how gp120 exerts its inhibitory effect, we verified whether gp120 was preventing the formation of blasts normally induced by antigenic stimulation. Clones expressing wild type CD4 were stimulated with antigen in the presence or absence of gp120 for 24 hours, and their size was monitored using the FACScan. As a control, an aliquot of cells were incubated without antigen or IL-2 for the same period. As depicted in figure 3A, the presence of gp120 prevented the formation of blasts in response to antigenic stimulation. In the lower profiles of figure 3, are depicted FCS/SSC contour blots of total cells (panel B, C, D) or with dead cells gated out using propidium iodide (panel E, F, G). In the lower left quadrant of each blot are the splenocytes used to present antigen. Panel B and E show that cells stimulated with antigen blast and are alive. On the

other hand, cells stimulated by antigen in the presence of gp120 did not increase in size (panel C). Furthermore, propidium iodide staining reveals that these cells are dead (panel F). These profiles are very reminiscent of what is observed in samples which were incubated without any antigen or IL-2 (panel D and G).

This T cell clone is dependent on exogenous IL-2 for growth and upon deprivation of IL-2, cells undergo apoptosis (data not shown). So the cell death observed in the presence of gp120 may be due either to a direct effect of gp120 on T cells or to a lack of stimulation and the absence of IL-2, leading to apoptosis.

gp120 inhibition is reversible by soluble CD4

To determine if the cell death observed in the presence of gp120 was due directly to the binding of gp120 to CD4, we verified whether soluble CD4 could reverse the inhibition induced by gp120. As shown in figure 4A, soluble CD4 completely prevented inhibition of antigenic stimulation by gp120 when added at the onset of stimulation. Interestingly, soluble CD4 completely restored antigen induced proliferation when added 6 hours after start of stimulation and partially restored the response when added 18 and 24 hours after start of stimulation. This partial reversal of inhibition cannot be due to lack of antigen, since addition of fresh splenocytes and antigen with the soluble CD4 at 24 hours still does not restore totally the response (data not shown).

These results argue against a direct cytopathic effect of gp120 on T cells but rather support the hypothesis that gp120 prevents the initiation of T cell activation without affecting viability. The observed cell death would thus be due to a consequent lack of IL-2 leading to apoptosis. Thus, the state of non-responsiveness induced by gp120 is reversible by treatment with soluble CD4 suggesting that cells exposed to gp120 have no long term memory of this treatment.

gp120 presence is required at G0/G1 phase of the cell cycle

In light of the above described results in which soluble CD4 could still restore partially the stimulation when added 24 hours after the onset of stimulation, we were interested in determining the time frame in which gp120 had to be present during stimulation to inhibit T cell activation.

As demonstrated in figure 4B, gp120 inhibited the response of clones expressing wild type or mutant forms of CD4 when added at the onset of stimulation. Surprisingly, when gp120 was added at 24 hours post-stimulation, we could still observe an inhibition of antigenic stimulation. We thus hypothesized that at the beginning of stimulation, cells are distributed in all phases of the cell cycle. The cells which are in the S, G2 and M phases at the onset of stimulation, must thus complete their cycle back to G0 before being stimulated with antigen and being susceptible to inhibition by gp120.

To verify that hypothesis, we synchronized CD4 positive cells in G0/G1 and compared their susceptibility to inhibition by gp120 to that of unsynchronized population. To synchronize cells, we first starved them of IL-2 for two hours, and then let them grow 12-16 hours in limiting amounts of IL-2 before stimulation. As shown in figures 5A and 5B, we routinely could obtain a population which is between 75% and 80% in the G0/G1 phase of the cell cycle as opposed to unsynchronized cells which were distributed more evenly in all phases of the cycle (40-45% in G0/G1). Both populations were stimulated with antigen and gp120 was added at different time points after the onset of stimulation (figure 5C). gp120 inhibited totally stimulation of both populations when added at start of stimulation. But addition of gp120 9 hours after start of stimulation only inhibited 56% of the response of the synchronized population whereas it inhibited 95% of the stimulation of the unsynchronized population. Addition of gp120 at 24 hours post stimulation further differentiated the two populations. While we can observe a 75% inhibition in the unsynchronized population, the synchronized population was only weakly inhibited (13%) when gp120 was added 24 hours after the onset of stimulation. These results suggest that all synchronized cells have seen antigen and become committed for activation before the 24 hour time point and thus cannot be inhibited by addition of gp120 at that time point. On the contrary, in the unsynchronized population, some cells are not stimulated yet at the 24 hour time point. These cells are the one that were in the S/ G2/M at time 0 and are now in G0/G1 and susceptible to inhibition by gp120 24 hours after start of stimulation. These results support the hypothesis that gp120 must be present in the G0/G1 phase of the cycle to inhibit antigenic stimulation.

Overall, we have demonstrated that inhibition of antigenic stimulation by gp120 is not mediated through direct activation of the CD4-associated lck and occurs in the absence of lck association with CD4. We propose that gp120 modulates in a reversible manner, a regulatory function of CD4 which occurs in the G0/G1 phase of the cell cycle and which is mapped to its extracellular domain.

Acknowledgments

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6. References

1. Banda, N. K., J. Bernier, D. K. Kurahara, R. Kurrle, W. Haigwood, R. P. Sekaly, and T. Finkel. 1992. Crosslinking CD4 by human immunodeficiency virus gp120 primes T cells for activation-induced apoptosis. J. Exp. Med. 176:1099-1106.

2. Blue, M.-L., D. A. Hafler, K. A. Craig, H. Levine, and S. F. Schlossman. 1987. Phosphorylation of CD4 and CD8 molecules following T cell triggering. Journal of Immunology 139:3949-3954.

3. Cefai, D., M. Ferrer, N. Serpente, T. Idziorek, A. Dautry-Varsat, P. Debre, and G. Bismuth. 1992. Internalization of HIV glycoprotein gp120 is associated with down modulation of membrane CD4 and $p56^{lck}$ together with impairment of T cell activation. J. Immunol. 149:285

4. Chimurle, N., V. S. Kalyanaraman, N. Oyaizu, H. B. Slade, and S. Pahwa. 1990. Inhibition of functional properties of tetanus antigen-specific T cell clones by envelope glycoprotein gp120 of human immunodeficiency virus. Blood 75:152

5. Chu, K. and D. R. Littman. 1994. Requirement for kinase activity of CD4-associated $p56^{lck}$ in antibody-triggered T cell signal transduction. Journal of Biological Chemistry 269:24095-24101.

6. Dianzani, U., U. Redoglia, F. Malavasi, M. Bragardo, A. Pileri, C. A. Janeway, and K. Bottomly. 1992. Isoform-specific associations of CD45 with accessory molecules in human T lymphocytes. Eur. J. Immunol. 22:365-371.

7. Glaichenhaus, N., N. Shastri, D. R. Littman, and J. M. Turner. 1991. Requirement for association of $p56^{lck}$ with CD4 in antigen-specific signal transduction in T cells. Cell 64:511-520.

8. Goldman, F., W. A. Jensen, G. L. Johnson, L. Heasley, and J. C. Cambier. 1994. gp120 ligation of CD4 induces $p56^{lck}$ activation and TcR desensitization independent of TcR tyrosine phosphorylation. J. Immunol 153:2905-2917.

9. Hall, C. G., J. Sancho, and C. Terhorst. 1993. Reconstitution of T cell receptor z-mediated calcium mobilization in non lymphoid cells. Science 261:915-917.

10. Haughn, L., S. Gratton, L. Caron, R. P. Sekaly, A. Veillette, and M. Julius. 1992. Association of tyrosine kinase $p56^{lck}$ with CD4 inhibits the induction of growth through the ab T - cell receptor. Nature 358:328-331.

11. Hurley, T. R., K. Luo, and M. Sefton. 1989. Activators of protein kinase C induce dissociation of CD4, but not CD8, from $p56^{lck}$. Science 245:407-409.

12. Iwashima, M., B. A. Irving, N. S van Oers, A. C. Chan, and A. Weiss. 1994. Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. Science 263:1136-1139.

13. Juszczak, R., H. Turchin, A. Truneh, J. Culp, and S. Kassis. 1991. Effects of human immunodeficiency virus gp120 glycoprotein on the association of the protein tyrosine kinase $p56^{lck}$ with CD4 in human T lymphocytes. J. Biol. Chem. 266:1

14. Kamitz, L., S. L. Sutor, T. Torigoe, J. C. Reed, M. P. Bell, D. J. McKean, P. J. Leibson, and R. T. Abraham. 1992. Effects of $p56^{lck}$ deficiency on the growth and cytolytic effector function of an interleukin-2-dependent cytotoxic T-cell line. Mol. Cell. Biol. 12:4521-4530.

15. Kornfeld, H., W. W. Cruikshank, S. W. Pyle, J. S. Berman, and D. M. Center. 1988. Lymphocyte activation by HIV-1 envelope glycoprotein. Nature 335:445-447.

16. Kupfer, A., S. J. Singer, C. J. Janeway, and S. L. Swain. 1987. Coclustering of CD4 (L3T4) molecule with the T-cell receptor is induced by specific direct interaction of helper T cells and antigen-presenting cells. Proc. Natl. Acad. Sci. USA. 84:5888-5892.

17. Luo, K. and B. M. Sefton. 1990. Cross-linking of T-cell surface molecules CD4 and CD8 stimulates phosphorylation of the lck tyrosine protein kinase at the autophosphorylation site. Mol. Cell. Biol. 10:5305-5313.

18. Mann, D. L., F. Lasane, M. Popovic, L. O. Arthur, W. G. Robey, W. A. Blattner, and M. J. Newman. 1987. HTLV-III large envelope protein (gp120) suppresses PHA-induced lymphocyte blastogenesis. J. Immunol. 138:2640

19. Mittler, R. S. and M. K. Hoffman. 1989. Synergism between HIV gp120 and gp120-specific antibody in blocking human T cell activation. Science 245:1380

20. Mittler, R. S., B. M. Rankin, and P. A. Kiener. 1991. Physical associations between CD45 and CD4 or CD8 occur as late activation events in antigen receptor-stimulated human T cells. Journal of Immunology 147:3434-3440.

21. Newell, M. K., L. J. Haughn, C. R. Maroun, and M. H. Julius. 1990. Death of mature T cells by separate ligation of CD4 and the T-cell receptor for antigen. Nature 347:286-289.

22. Oyaizu, N., N. Chirmule, V. S. Kalyanaraman, W. W. Hall, R. A. Good, and S. Pahwa. 1990. Human immunodeficiency virus type 1 envelope glycoprotein gp120 produce immune defects in CD4+ T lymphocytes by inhibiting interleukin-2 mRNA. Proc. Natl. Acad. Sci. USA 87:2379

23. Pelchen-Matthews, A., I. J. Parsons, and M. Marsh. 1993. Phorbol ester-induced downregulation of CD4 is a multistep process involving dissociation from $p56^{lck}$, increased association with clathrin-coated pits and altered endosomal sorting. J. Exp. Med. 178:1209-1222.

24. Perlmutter, R. M. 1990. Translational regulation of the lymphocyte-specific protein tyrosine kinase $p56^{lck}$. Enzyme 44:214-224.

25. Rosoff, P. M., S. J. Burakoff, and J. L. Greenstein. 1987. The role of L3T4 molecule in mitogen and antigen activated signal transduction. Cell 49:845-853.

26. Rudd, C. E., J. M. Trevillyan, J. V. Dasgupta, L. L. Wong, and S. F. Schlossman. 1988. The CD4 antigen is complexed in detergent lysates tom protein tyrosin kinase (pp58) from human T lymphocytes. Proc. Natl. Acad. Sci. USA 85:5190

27. Sakihama, T., A. Smolyar, and E. L. Reinherz. 1995. Oligomerization of CD4 is required for stable binding to class II MHC proteins but not for interaction with HIV gp120. Proc. Natl. Acad. Sci. USA 92:6444-6448.

28. Shaw, A. S., K. E. Amrein, C. Hammond, D. F. Stern, B. M. Sefton, and J. K. Rose. 1989. The cytoplasmic domain of CD4 interacts with the tyrosine protein kinase, $p56^{lck}$, through its unique amino-terminal domain. Cell 59:627

29. Shaw, A. S., J. Chalupny, A. Whitney, C. Hammond, K. E. Amrein, P. Kavathas, B. M. Sefton, and J. K. Rose. 1990. Short related sequences in the cytoplasmic domains of CD4 and CD8 mediated binding to the amino-terminal domain of the $p56^{lck}$ tyrosine protein kinase. Mol. Cell. Biol. 10:1853-1862.

30. Shin, J., C. Doyle, Z. Yang, D. Kappes, and J. Strominger. 1990. Structural features of the cytoplasmic region of CD4 required for internalization. EMBO Journal 9:425-434.

31. Sleckman, B. P., J. Shin, V. E. Igras, T. L. Collins, J. L. Strominger, and S. J. Burakoff. 1992. Disruption of the CD4-$p56^{lck}$ complex is required for rapid internalization of CD4. Proc. Natl. Acad. Sci. USA 89:7566-7570.

32. Soula, M., R. Fagard, and S. Fisher. 1992. Interaction of human immunodeficiency virus glycoprotein gp160 with CD4 in Jurkat cells increases $p56^{lck}$ autophosphorylation and kinase activity. Int. Immunol. 4:295

33. Strauss, B. and A. Weiss. 1992. Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor. Cell 70:585-593.

34. Tite, J. P., A. Sloan, and C. A. Janeway. 1986. The role of L3T4 in T-cell activation: L3T4 may be both an Ia binding protean and receptor that transduces a negative signal. J. Mol. Cell. Immunol. 2:179

35. Turner, J. M., M. H. Brodsky, B. A. Irving, S. D. Levin, R. M. Perlmutter, and D. R. Littman. 1990. Interaction of the unique N-terminal region of tyrosine kinase $p56^{lck}$ with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. Cell 60:755-765.

36. Veillette, A., M. A. Bookman, E. M. Horak, and J. B. Bolen. 1988. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase $p56^{lck}$. Cell 55:301-308.

37. Veillette, A., M. A. Bookman, E. M. Horak, L. E. Samelson, and J. B. Bolen. 1989. Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase $p56^{lck}$. Nature 338:257

38. Weyand, C. M., J. Goronzy, and G. Fathman. 1987. Modulation of CD4 by antigenic activation. J. Immunol 138:1351-1354.

39. Zerbib, A. C., A. B. Reske-Kunz, P. Lock, and R. P. Sekaly. 1994. CD4-mediated enhancement or inhibition of T cell activation does not require the CD4:$p56^{lck}$ association. Journal of Experimental Medicine 179:1973-1983.

Figure legends

Figure 1

A) gp120 inhibits antigenic stimulation in a dose dependent fashion. $5X10^4$ T cells were incubated with $5X10^5$ irradiated C57/Bl6 splenocytes and 200 µg/ml of ovalbumin, in the presence or absence of gp120 at the indicated concentrations, for 40 hours at 37°C. Cells were then pulsed with 1 µcurie of ^{3}H-thymidine for 6 hours before harvesting. B) gp120 inhibits activation by a wide range of antigen concentrations. $5X10^4$ T cells were incubated with $5X10^5$ irradiated C57/Bl6 splenocytes and ovalbumin at the indicated concentrations, in the presence (gp120) or absence (NS) of 5 µg/ml gp120, for about 40 hours at 37 °C. Cells were then pulsed with 1 µcurie of ^{3}H-thymidine for 6 hours before harvesting.

Figure 2

A) gp120 inhibits stimulation of C4202A and CD4 3S clones. $5X10^4$ T cells were incubated with $5X10^5$ irradiated C57/BL6 splenocytes and 200 µg/ml of ovalbumin, in the presence (gp120) or absence (NS) of gp120 at 5 µg/ml, for 40 hours at 37°C. Cells were then pulsed with 1 µcurie of ^{3}H-thymidine for 6 hours before harvesting. B) EGF does not inhibit stimulation of chimera expressing clones. $5X10^5$ of either EC1 or EC4 T cells which express the EGFRCD4 chimera were stimulated with 100 µg/ml ovalbumin presented by $5X10^5$ irradiated C57/BL6 splenocytes, in the presence of EGF (UBI) at 100 nM or media (NS), for 40 hours at 37°C. Cells were then pulsed with 1 µcurie of ^{3}H-thymidine for 6 hours before harvesting.

Figure 3

gp120 prevents blasting of cells and leads to cell death. Cells were stimulated with ovalbumin at 200 µg/ml (B, C, E, F) or media (D, G) in the presence of gp120 at 5 µg/ml (C, F) or media (B, E, D, G) and irradiated C57/Bl6 splenocytes for 24 hours. Cells were harvested and analyzed using the FACscan. Panel A depicts the FSC profiles of cells stimulated with antigen only (light line) and cells stimulated with antigen in the presence of gp120 (dark line). Panels B, C and D show the FSC/SSC profiles of total cells. Panels E, F, G show the FSC/SSC profiles of populations in which dead cells are gated out using propidium iodide.

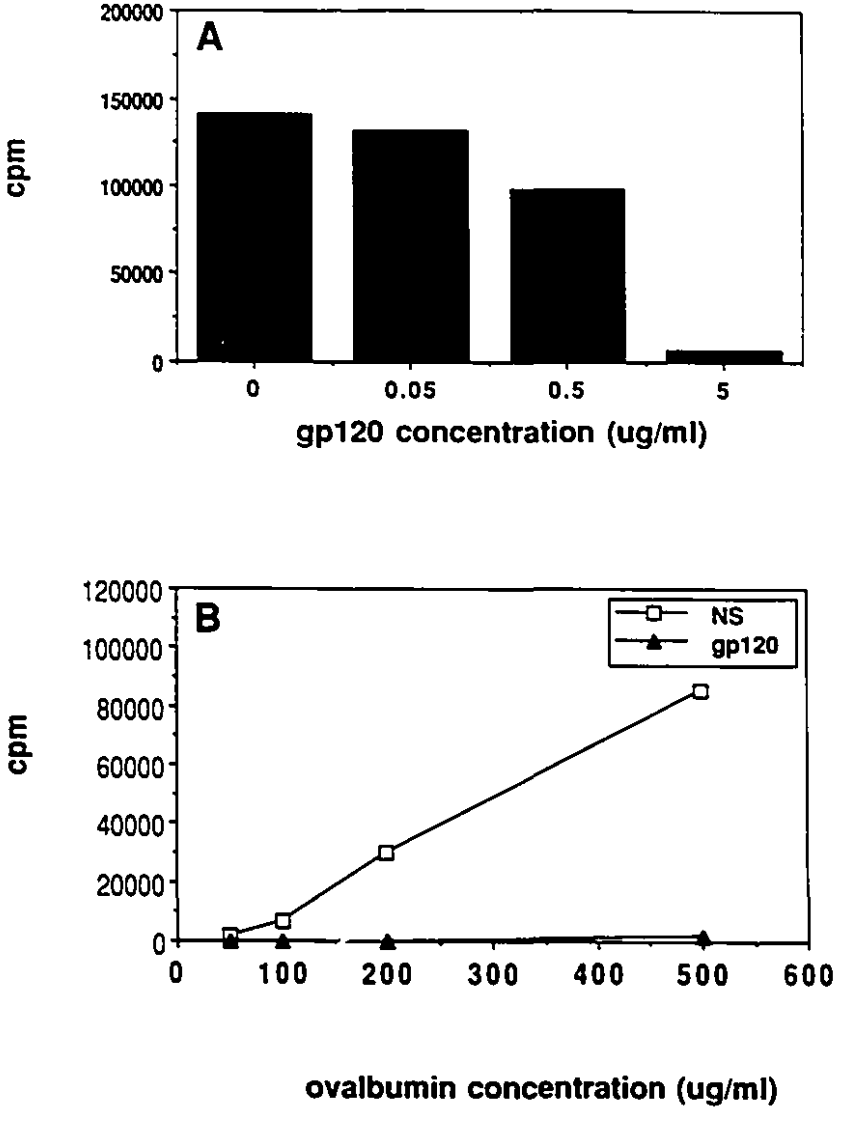
Figure 4

A) Soluble CD4 reverses the inhibition of stimulation induced by gp120. Cells expressing wild type CD4 were stimulated with ovalbumin at 200 µg/ml presented by C57/Bl6 irradiated splenocytes in the presence (gp120) or absence (NS) of gp120 at 5 µg/ml. Soluble CD4 at 5 µg/ml was added at 0, 6, 18 or 24 hours post-stimulation as indicated in the legend. Total stimulation time was 40 hours before ^{3}H-thymidine incorporation. B) Time course of addition of gp120. Cells expressing different CD4 constructs were stimulated with ovalbumin at 100 µg/ml. gp120 was added at 5 µg/ml at start of stimulation or 24 hours later. ^{3}H-thymidine incorporation was performed 40 hours after start of stimulation.

Figure 5

A) and B) Cell cycle analysis of unsynchronized (panel A) and synchronized (panel B) wild type CD4 expressing cells. Cells were stained using Krishan buffer as described in material and methods and analyzed using the FACSTAR plus (Becton Dickinson). C) Time course of addition of gp120. Synchronized and unsynchronized cells were stimulated with ovalbumin at 200 µg/ml only (NS) or with addition of gp120 at 5 µg/ml at 0, 9 or 24 hours post stimulation. Total stimulation length is 40 hours before a 6 hour pulse with ^{3}H-Thymidine.

FIGURE 1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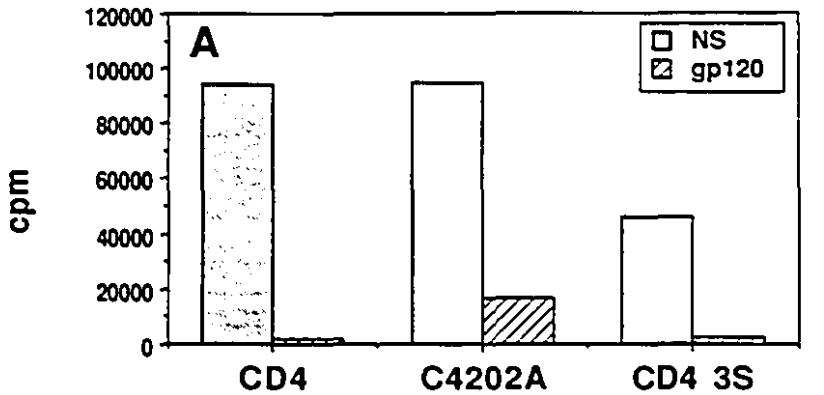
ovalbumin concentration (ug/ml)

FIGURE 2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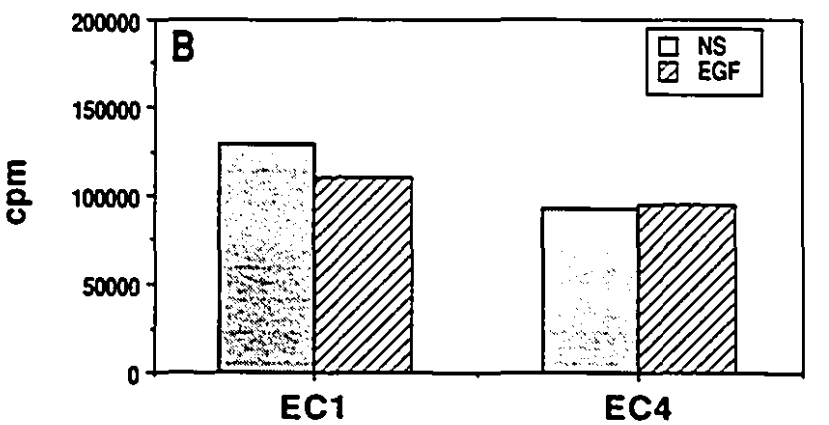
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FIGURE 3

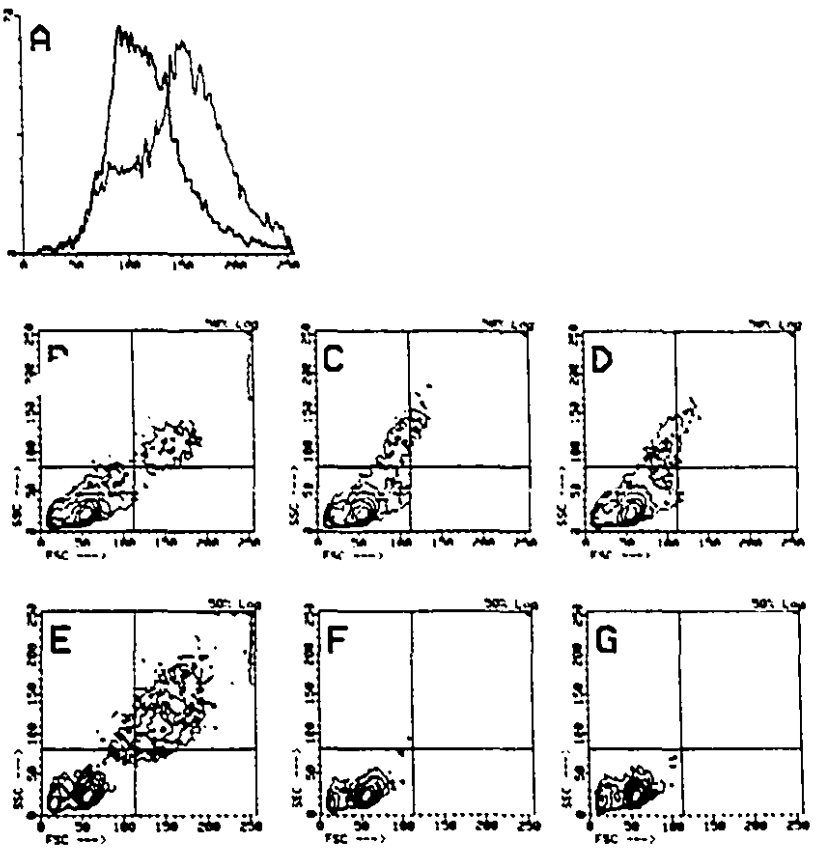

FIGURE 4

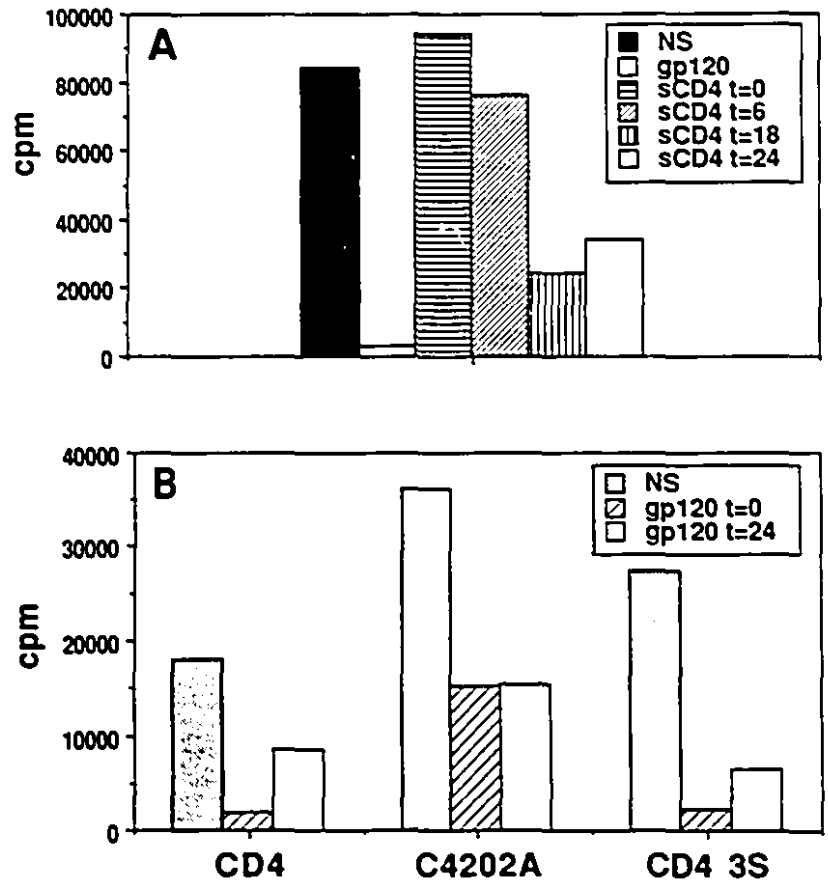
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FIGURE 5

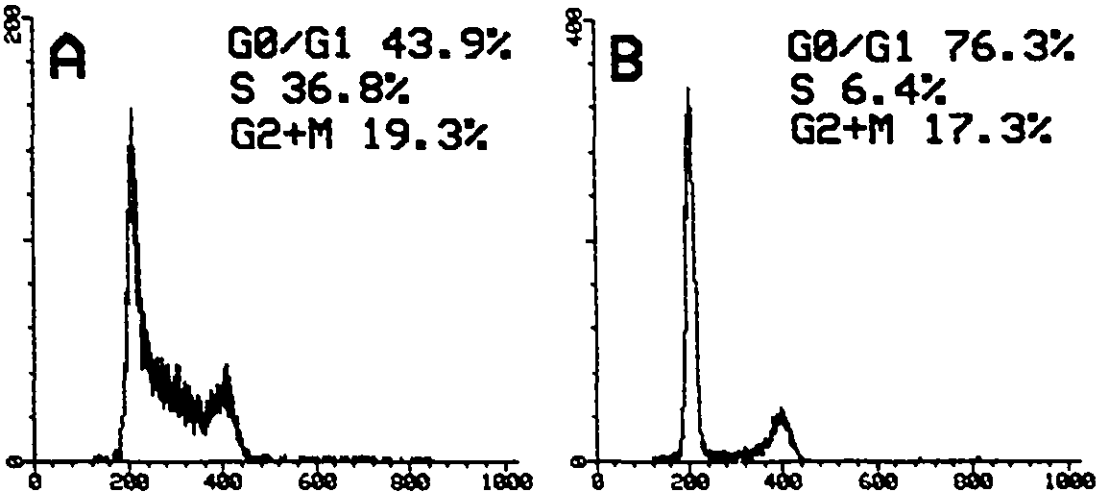
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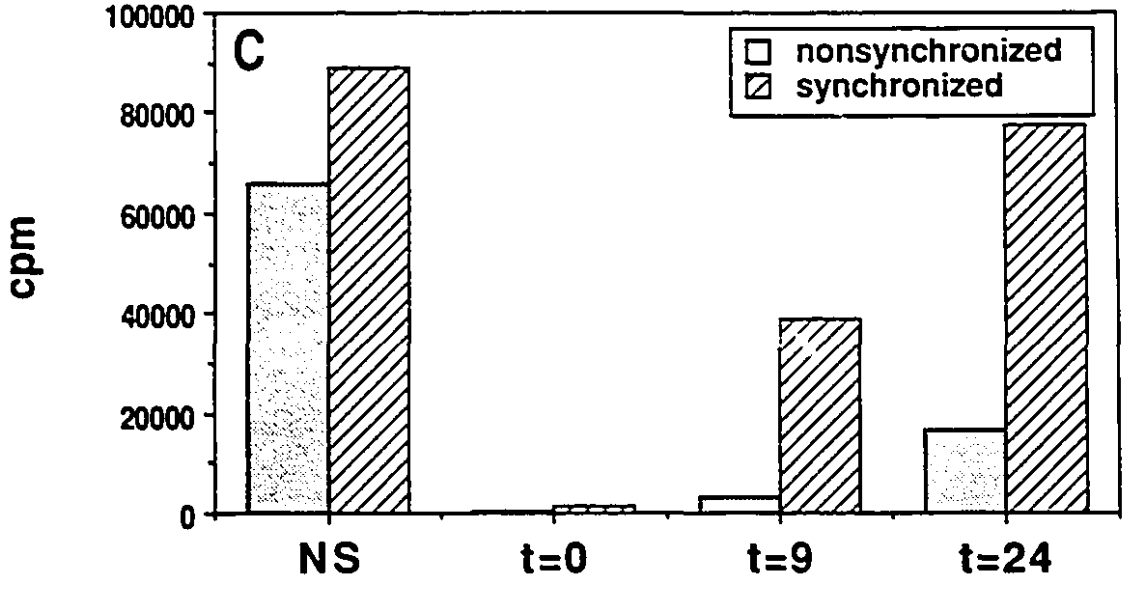
FIGURE 5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CHAPTER 5

ASSOCIATION OF p56^{lck} TO THE CYTOPLASMIC DOMAIN OF CD4 MODULATES HIV-1 EXPRESSION

1. Preface

The last chapter described how gp120 binding to CD4 could modulate T cell activation. Since both HIV and the IL-2 promoter share a number of transcription factors, we thus hypothesized that gp120 binding to CD4 could also modulate HIV infection. To verify if the CD4/lck association could send signals to the HIV promoter, we transfected two human T cell lines with either wild type CD4 or mutants of CD4 that have lost the ability to associate with lck. These cell lines were then infected with different HIV isolates and levels of viral replication were compared between cell lines expressing wild type CD4 and mutants of CD4.

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Association of p56^{lck} with the cytoplasmic domain of CD4 modulates HIV-1 expression

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To investigate the role played by the cytoplasmic domain of the CD4 glycoprotein in the process of HIV infection, we have transfected two CD4-negative human T cell lines with cDNAs encoding the full-length CD4 and a truncated form of the molecule, lacking most of the cytoplasmic domain. Levels of viral replication were significantly higher in cells carrying the truncated version of CD4, in comparison with cells expressing the full-length CD4, as measured by the percentage of cells expressing viral p24 protein and the number of infectious particles released into culture supernatants. The extent of viral entry and reverse transcription was similar in each case, as monitored by an enzymatic test and quantitative PCR. Quantitative differences at RNA and protein levels were responsible for changes in viral production. To further characterize the mechanisms responsible for decreased rates of HIV replication in CD4-expressing cells we have treated the different cell lines, very early after HIV infection, with azidothymidine and soluble CD4, two antiviral agents that inhibit replication of HIV at different stages in the virus replicative cycle. Results from these experiments indicate that a cellular signal is mediated by the CD4 molecule, which negatively regulates the expression of viral DNA already present in such cells. This signal would be initiated following oligomerization of the CD4 molecule by the virus itself. Results from experiments with a CD4 construct containing mutations of the cysteine residues which are responsible for association of CD4 with p56^{lck} demonstrate that p56^{lck} is implicated in the transduction of the signal negatively regulating HIV replication.

Key words: AIDS/CD4/HIV

Introduction

The CD4 molecule is a 55 kDa surface protein that contains four extracellular domains that have structural homology with an immunoglobulin V region (Ryu *et al.*, 1990; Wang *et al.*, 1990). The CD4 glycoprotein is highly expressed on T cells involved in recognition of antigen in the context of class II major histocompatibility complex (MHC) molecules (Swain, 1983). Interaction of CD4 with a non-polymorphic region of class II molecules was reported to enhance the avidity between the T cell receptor and its ligands (Gay *et al.*, 1987; Sleckman *et al.*, 1988; Lamarre *et al.*, 1989). Data suggesting that the CD4 molecule is directly involved in signal transduction came from numerous reports. Negative signaling via CD4 was reported to occur based on the fact that some anti-CD4 monoclonal antibodies (mAbs) can inhibit T cell activation in the absence of accessory molecules (Wilde *et al.*, 1983; Bank and Chess, 1985; Geppert and Lipsky, 1987).

The observation that CD4 is physically associated with the tyrosine protein kinase p56^{lck} further reinforced the notion that this integral membrane glycoprotein participates in signal transduction (Rudd *et al.*, 1988; Veillette *et al.*, 1988). p56^{lck} is a member of the *src* family of cytosolic protein tyrosine kinases and is expressed predominantly in T lymphocytes (Rudd *et al.*, 1988; Veillette *et al.*, 1988). Cross-linking of CD4 with CD4-specific mAbs induces a rapid increase in the autophosphorylation state and in the kinase activity of p56^{lck} (Veillette *et al.*, 1989; Koretzky *et al.*, 1990; Luo and Sefton 1990). Mutations in CD4 that abolish the interaction with p56^{lck} have led to decreased IL-2 secretion following antigenic stimulation, clearly indicating that this molecule plays an important role in T cell activation (Glaichenhaus *et al.*, 1991). Moreover, the ability of a T cell hybridoma to secrete IL-2 in response to antigen stimulation was increased following the introduction of an active form of p56^{lck} (Abraham *et al.*, 1991). The essential function of p56^{lck} in T cell ontogeny was also illustrated using *lck*-deficient mice in which no mature functional T lymphocytes could be detected (Molina *et al.*, 1992). Altogether these studies indicate that the CD4 molecule plays an important role in T cell activation through its association with p56^{lck}.

The CD4 molecule is the primary cellular receptor for the human immunodeficiency virus (HIV) due to the high-affinity binding of the external viral envelope protein (gp120) to CD4 (reviewed in Sattentau and Weiss, 1988). The binding site of gp120 has been mapped to the N-terminal extracellular region of CD4 and more precisely within the first immunoglobulin-like domain termed D_1 (Landau *et al.*, 1988; Mizukami *et al.*, 1988; Peterson and Seed, 1988; Arthos *et al.*, 1989; Fleury *et al.*, 1991). The cytoplasmic domain of CD4 is not required for HIV-1 entry into cells since infection was shown to occur despite the deletion of the whole domain (Bedinger *et al.*, 1988; Maddon *et al.*, 1988). The objective of these studies was to clarify the precise role played by the cytoplasmic domain of CD4 in the process of HIV infection. We demonstrate that the intracellular domain of CD4 negatively regulates the replicative rate of HIV-1 in T-lymphoid cells. Most

importantly, the association of CD4 with the protein tyrosine kinase p56^{lck} is shown to be required for this effect.

Results

Expression of wild-type, truncated and mutated forms of CD4 in A2.01 and HSB-2 cells

We have expressed in two CD4-negative T cell lines (A2.01 and HSB-2) wild-type (wt-CD4), truncated (t-CD4) and a mutated form of CD4 with substitution of cysteine residues 420 and 422 by alanine residues (C420,422A). Independently derived clones of each construct were obtained and expression of CD4 was assessed by flow cytometry (Figure 1). It is of interest to note that higher levels of CD4, on a greater percentage of cells, were detected in clones expressing wt-CD4 when compared with cells transfected with t-CD4 or C420,422A. The affinity of wild-type and truncated CD4 for radiolabeled gp120 was measured by a quantitative gp120 binding assay and was found to be similar in the various clones tested (data not shown), thereby indicating that deletion of most of the cytoplasmic domain of CD4 did not alter its affinity for gp120.

Infection of A2.01 and HSB-2 cells expressing wt-CD4 and t-CD4 with HIV-1

A2.01 cells expressing either wt-CD4 or t-CD4 were infected with the HIV-IIIB laboratory strain of HIV-1 at a multiplicity of infection (m.o.i.) (infectious virus/target cell) of 0.01. The percentage of p24-expressing cells was evaluated by indirect immunofluorescence. Three independently derived clones, obtained from the same transfection, expressing either wt-CD4 or t-CD4, were studied. These experiments consistently showed that a greater number of cells expressing specific viral p24 antigen was detected in cell lines carrying t-CD4 (Figure 2A) at 12 days post-infection. A 12- to 40-fold increase in the percentage of cells expressing p24 was noted in the three cell lines expressing t-CD4 as compared with cells transfected with wt-CD4. A 30- to 300-fold increase in the release of infectious viral particles was detected in culture supernatants originating from cells carrying t-CD4 as compared with cells transfected with wt-CD4 following HIV-1 infection (Figure 2B). These experiments were repeated several times using these different clones and consistently showed that cells expressing the truncated form of CD4 yielded faster kinetics of HIV-1 infection. The most prominent difference in the level of HIV-1 replication was observed between clones A2D8 and 3D4D8 transfected with wt-CD4 and t-CD4, respectively. Most of our subsequent experiments were performed using these two clones. Similar differences at the level of virus production were obtained in subsequent experiments performed using various m.o.i. ranging from 0.01 to 0.14 (Figure 2C). To validate our results further, other cellular clones were derived from an independent transfection. Again, a marked increase in the level of HIV-1 replication was observed in t-CD4 cells as compared with wt-CD4 cells (data not shown). To confirm that this observation was not strain specific, these same cells were infected with the SF-2 strain of HIV-1 (Cheng-Mayer and Levy, 1988). A 30-fold increase in the percentage of cells expressing viral p24 antigen was detected in t-CD4 cells as compared with wt-CD4 cells (Figure 2D). To confirm that the role of the cytoplasmic tail of CD4 in HIV-1 replication

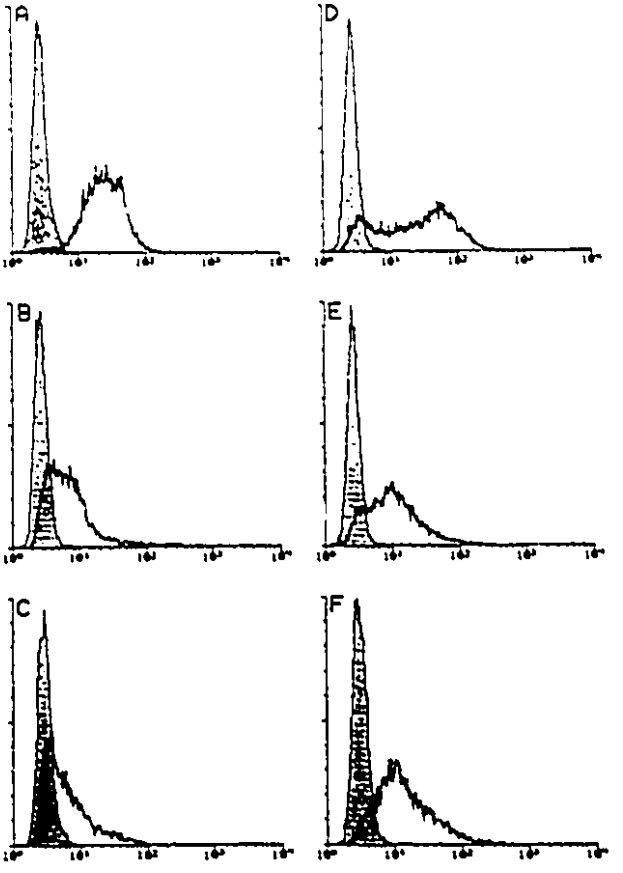

Fig. 1. Flow cytometry analysis of CD4 expression in cell lines transfected with various CD4 constructs. Cells were incubated with CD4-specific mAb 1F3 prior to staining with FITC-conjugated goat anti-mouse antibody. (A) A2.01 cells transfected with wt-CD4 (clone A2D8). (B) A2.01 cells carrying t-CD4 (clone 3D4D8). (C) A2.01 cells expressing CD4 mutated at positions 420 and 422. (D) HSB-2 cells transfected with wt-CD4 (clone A4). (E) HSB-2 cells carrying t-CD4. (F) HSB-2 cells carrying CD4 mutated at positions 420 and 422 (clone 8).

was not restricted to A2.01 cells, the same CD4 constructs (wt-CD4 and t-CD4) were also expressed in HSB-2, another CD4-negative cell line. Again, the replication of HIV-1 was reproducibly greater in cells expressing the truncated form of CD4 with a 4- to 11-fold increase in the percentage of p24-positive cells observed at 15 days post-infection (Figure 2E). Moreover, a 6-fold increase in the number of infectious viral particles was detected in culture supernatants originating from infected cells carrying t-CD4 in comparison with cells transfected with wt-CD4 (Figure 2F). These results indicate that the cytoplasmic domain of CD4 can modulate HIV-1 replication. More importantly, this observation is not restricted to a specific virus strain or T cell line.

Qualitative differences in viral proteins are not responsible for changes in virus replication

To determine whether differences in the levels of viral replication were associated with qualitative and/or quantitative defects of specific viral proteins, Western blot analysis was performed on infected cell lines using antisera from infected patients (Figure 3A). Similar amounts of total cellular lysates obtained from A2D8 and 3D4D8 cells infected with HIV-1 for 7, 12 and 15 days were analyzed.

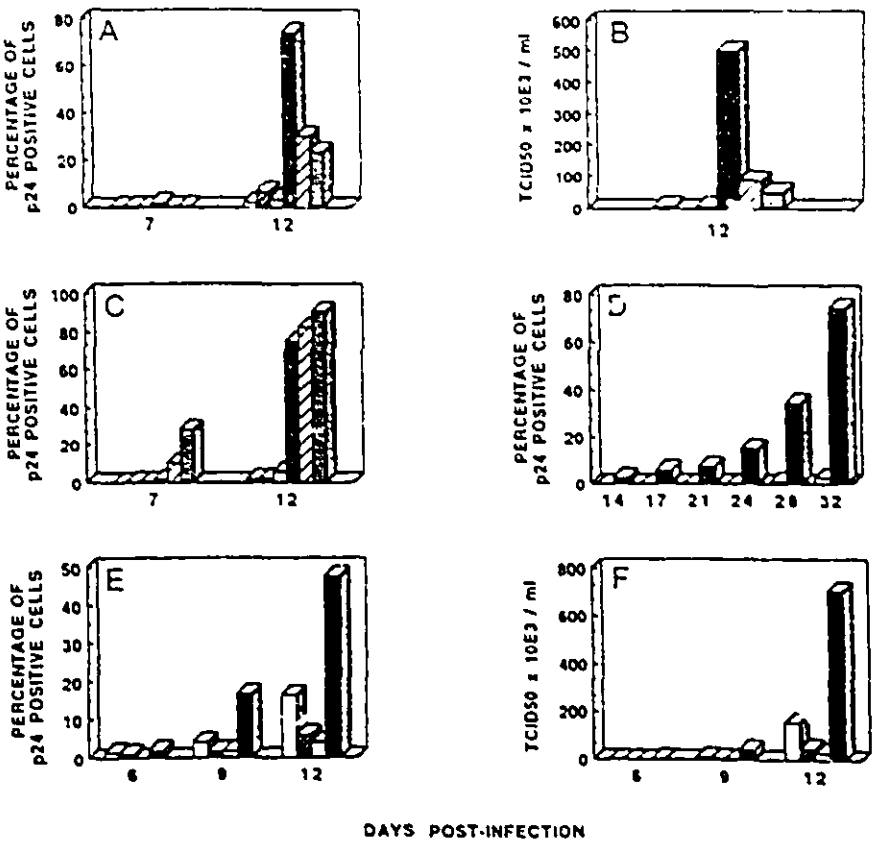

Fig. 2. Infection of transfected cells with HIV-1. (A) Percentage of p24-positive cells after infection of transfected A2.01 cells with HIV-1. Independently derived clones (5×10^5 cells) carrying either wt-CD4 (A2D8, □; A2A5, ▨; C4B9, □) or t-CD4 (3D4D8, ■; D4B11, ▨; D4F8, ■) were incubated with HIV-IIIB (m.o.i., 0.01) for 90 min at 37°C. Cells were washed twice with PBS and were resuspended in fresh complete RPMI. Determination of viral p24-expressing cells was by indirect immunofluorescence at 7 and 12 days after viral infection. (B) Number of infectious viral particles following HIV-1 infection of wt-CD4 and t-CD4 A2.01 cells. Supernatants from cells infected with HIV-IIIB (12 days post-infection) were clarified by centrifugation and $TCID_{50}$ was determined using MT-4 cells as described in Materials and methods. (C) Percentage of p24-positive cells following infection of transfected A2.01 cells with HIV-1 at various m.o.i. Cells were infected with HIV-IIIB at a m.o.i. of 0.01 (A2D8, □); (3D4D8, ■), 0.07 (A2D8, ■); (3D4D8, ▨) or 0.14 (A2D8, ▨); (3D4D8, ▨). (D) Percentage of p24-positive cells following infection with the SF-2 strain of HIV-1. A2.01 cells carrying either wt-CD4 (A2D8) (□) or t-CD4 (3D4D8) (■) were infected with SF-2 (m.o.i., 0.14). (E) Percentage of p24-positive cells after infection with HIV-1 (m.o.i., 0.03) of HSB-2 cells transfected with wt-CD4 (A1, □; A4, ■; A7, ▨) and t-CD4 (■). (F) Number of infectious viral particles following infection of HSB-2 cells carrying wt-CD4 (A1, □; A4, ▨; A7, ▨) and t-CD4 (■).

Controls consisted of uninfected A2D8 and 3D4D8 cells, and of U937 cells chronically infected with HIV-IIIB. All major structural HIV-1 proteins (gp120, p66, p55, gp41 and p24) were detected in chronically infected U937 cells. These specific viral proteins were detected in 3D4D8 cells as early as 7 days following HIV-1 infection. In contrast, viral proteins were observed in A2D8 cells only 15 days following HIV-1 infection. A time-dependent increase in the amount of viral proteins was observed in both cell lines. These experiments clearly demonstrated the absence of qualitative changes in viral protein synthesis despite a marked increase in the synthesis of major structural viral proteins in cells carrying t-CD4 as compared with cells transfected with wt-CD4. These results are in agreement with those obtained with indirect immunofluorescence (percentage of p24-positive cells) and co-culture (number of infectious particles in culture supernatants) assays.

Quantitative differences in HIV-1 RNA directly correlate with levels of HIV-1 proteins

Since we observed marked differences in the expression of viral proteins, it was important to evaluate if such changes were associated with different RNA levels. For this purpose, Northern blot analysis of A2D8 and 3D4D8 RNA was carried out at different times after HIV-1 infection using a DNA probe which detects the three specific viral RNA species (Figure 3B, upper panel). Chronically infected U937 cells, used as control, express all three specific RNA bands. The full-length unspliced 9.2 kb mRNA encompasses the viral genomic mRNA and the mRNA for the *gag-pol* and *gag* proteins; the intermediate (−4−5 kb) corresponds to *vpu*, *env*, *vif*, *vpr* and *tat* mRNAs, while the small multiply spliced (−2 kb) mRNA produces the regulatory proteins *tat*, *rev* and *nef*. The full-length genomic RNA was detected in 3D4D8 cells as early as 7 days after viral infection. RNA levels increased in a time-dependent manner. However, when A2D8 cells were analyzed with the same probe, it was not possible to detect any specific RNA before 12 days after infection with HIV-1. To quantitate the levels of viral RNAs more precisely, slot-blot analysis was performed using total RNA extracted from infected A2D8 and 3D4D8 cells, and from chronically infected U937 cells (Figure 3B, lower panel). Ratios of HIV-1 mRNA and actin mRNA clearly indicate a 4-fold increase in the levels of HIV-1 mRNA in cells expressing t-CD4 as compared with cells transfected with wt-CD4. Results from this analysis further supported

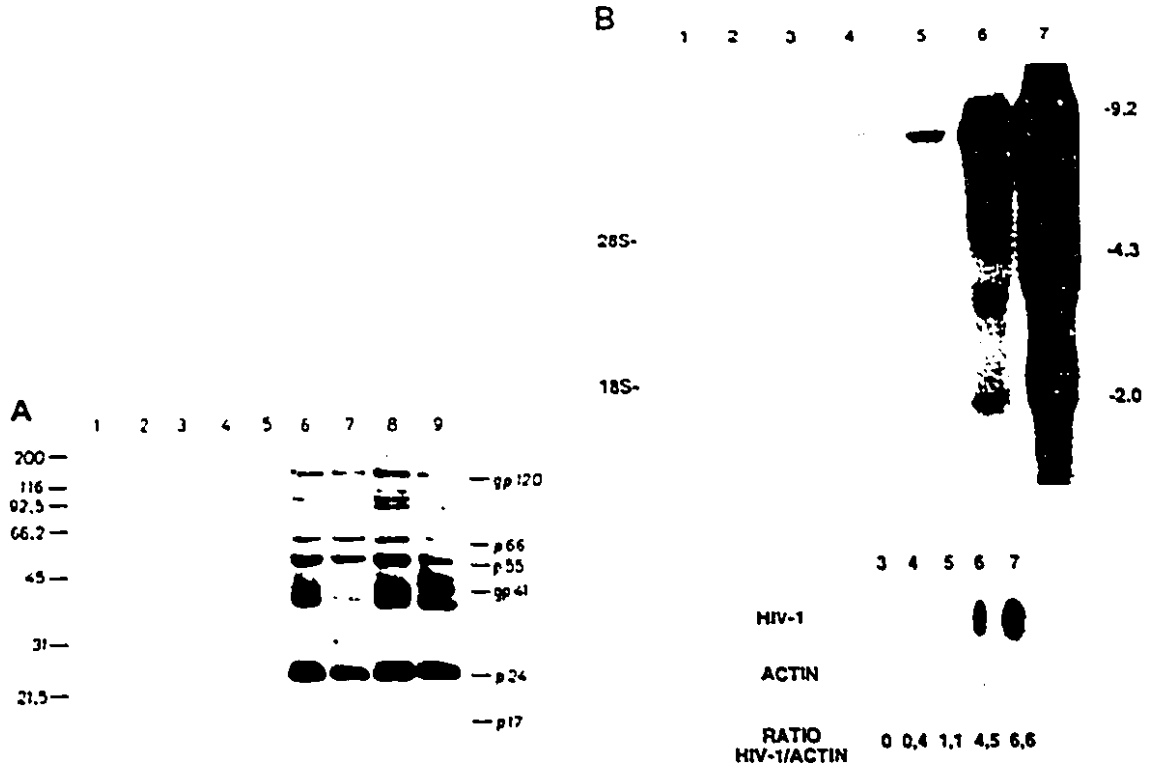

Fig. 3. (A) Western blot analysis of specific viral protein expression following HIV-1 infection of transfected A2.01 cells. Cellular lysates (100 μg) were resolved on a 10% polyacrylamide gel, transferred to nitrocellulose filter, incubated with polyclonal HIV-1-positive sera (1:500) and then with [^{125}I]recombinant protein A/G. Samples were from wt-CD4 A2.01 cells (A2D8) either uninfected (1) or at 7 (3), 12 (5) and 15 days post-infection (7) and from t-CD4 A2.01 cells (3D4D8) either uninfected (2) or at 7 (4), 12 (6) and 15 (8) days post-infection. Chronically HIV-IIIB-infected U937 cells served as control (9). (B) Upper panel: Northern blot analysis of total RNA (10 μg) from cells infected with HIV-1 using pBH-10 (HIV-1) as probe. Lower panel: quantification of RNA content by slot-blot analysis. Total RNA (1.25 μg) was hybridized to pBH-10 and mouse actin probes, respectively. Samples were from A2.01 cells carrying wt-CD4 (A2D8) at 7 days (3) and 12 days post-infection (5) or A2.01 cells expressing t-CD4 (3D4D8), at 7 days (4) and 12 days post-infection (6), and U937 cells chronically infected with HIV-IIIB (7). Autoradiographs were analyzed using the RAS-1000 (Research Analysis System) image analysis system to evaluate HIV-1 and actin band intensity which are expressed in optical density (OD/pixels).

the observation that viral RNAs were more abundant in HIV-1 infected cells carrying t-CD4, as compared with cells expressing wt-CD4.

Cellular parameters and viral entry are similar in cells expressing wt-CD4 and t-CD4

Next, we started to examine which step in the virus replicative cycle was responsible for such marked changes in the levels of viral production between the two cell lines. Growth curves were determined for A2D8 and 3D4D8 cells, either infected or not with HIV-IIIB (m.o.i., 0.14). Doubling times were similar for each cell line and could not be responsible for the enhanced viral replication detected in cells expressing t-CD4 (data not shown). Cell viability, as assessed by Trypan blue exclusion, was also determined and the percentage of live cells up to 21 days post-infection was close to 100% for both cell lines (data not shown). Modulation of the CD4 molecule from the cell surface may also influence the rate of viral entry, thereby affecting levels of viral replication. For this purpose, A2D8 and 3D4D8 cells were incubated at 37°C with HIV-IIIB (m.o.i., 0.14) and CD4 surface expression was monitored at specific time points following viral infection. Results from these experiments demonstrated that the levels of CD4 expressed on the surface of cells transfected with wt-CD4 (A2D8) or t-CD4 (3D4D8) remained unchanged for 7 days post-infection. However, a more rapid down-modulation of CD4 expression was detected over longer periods of time on cells expressing t-CD4 which correlates with the higher rate of HIV-1 replication in such cells (data not shown).

Table I. Determination of initial viral entry into A2.01 cell lines using a p24 enzymatic assay

Cell line	p24 antigen level (pg/ml)[a]
A2D8 (control)	386 ± 93
A2D8 + HIV-1	6800 ± 141
3D4D8 (control)	254 ± 78
3D4D8 + HIV-1	3975 ± 106

Cells (2×10^6) expressing either wt-CD4 (A2D8) or t-CD4 (3D4D8) were incubated on ice with HIV-IIIB (m.o.i., 1.0) for 30 min and were subsequently incubated at 37°C for 90 min. The incubation period at 37°C was omitted for controls. Thereafter, cells were washed twice and incubated for 2 min on ice with 200 μl of complete RPMI medium at pH 3.0 to remove viruses that had not entered cells. Cells were washed twice with cold PBS prior to resuspension in 200 μl fresh RPMI supplemented with Triton X-100 (1%) and the p24 capture assay was performed according to the manufacturer's instructions. The incubation period at 37°C was omitted for controls.
[a]Results shown are the mean ± SD of three estimations.

Experiments aimed at quantitating the virus load entering cells very early after infection were also carried out. The

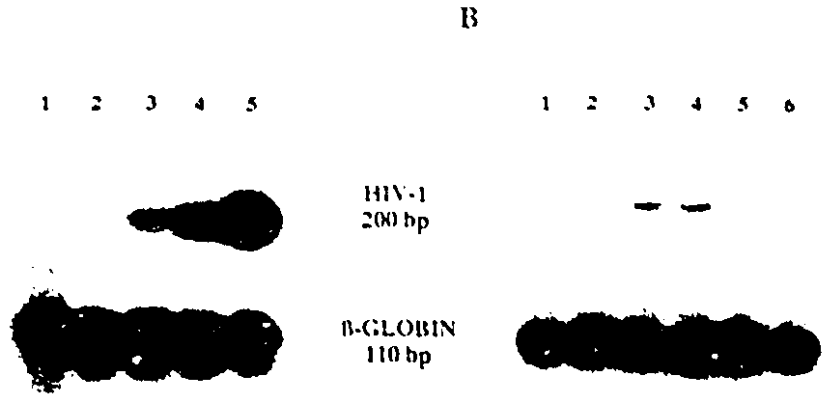

Fig. 4. PCR analysis of cellular HIV-1 DNA levels. PCR amplification of viral DNA was performed as described in Materials and methods using total cellular DNA. (A) Linearity of the PCR was evaluated using ACH-2 cells. To minimize variations in the efficiency of the PCR, samples were adjusted to 1 μg total DNA per sample using placental DNA (Sigma, MO). Samples originate from 0 (1), 10 (2), 100 (3), 1000 (4) and 10 000 (5) ACH-2 cells. (B) Cells expressing wt-CD4 (A2D8) (3) or t-CD4 (3D4D8) (4) were infected with HIV-IIIB (m.o.i., 1.0) and cultured for 6 h prior to DNA extraction. Amplified fragments were subjected to electrophoresis on a 6% non-denaturing gel and visualized by autoradiography of the gel. Two sets of primers were included in each tube: an HIV-1 specific primer pair called (M667/M661) and an oligonucleotide primer pair specific for human β-globin which served as an internal control. Ratios between amplified signal with the HIV-1 set of primers and the β-globin set were calculated for each experimental condition using image analysis system (RAS 1000). Uninfected cells (A2D8, lane 1; 3D4D8, lane 2) and cells (A2D8, lane 5; 3D4D8, lane 6) infected with the heat inactivated viruses (30 min at 56°C) were used as controls.

above-described cell lines were incubated with HIV-1 at 4°C to permit viral binding and were subsequently transferred at 37°C to allow viral entry. The cells were then incubated at pH 3.0 to remove viruses which had not entered cells. Levels of viral p24 protein as monitored using a commercial enzymatic assay (Table I), revealed that viral entry was not increased in cells carrying t-CD4. To quantitate precisely the extent of viral entry, semi-quantitative PCR was performed at 6 h after infection. To decrease PCR amplification of premature HIV-1 reverse transcripts, we have used a set of primers (M667/M661) that recognize only full-length or nearly completely synthesized viral DNA (Zack *et al.*, 1990). To rule out the possibility that PCR amplification could be due to the presence of partial reverse transcripts in infectious mature HIV-1 particles (Lori *et al.*, 1992; Trono, 1992), PCR amplifications were carried out on cells previously incubated with heat inactivated virus (Figure 4B, lanes 3 and 4). Linearity of the PCR assay was assessed by using different concentrations of ACH-2 cells previously reported to contain one copy of viral DNA (Figure 4A). Results of the experiments clearly indicated that increased viral replication in cells expressing the truncated form of CD4 could not be attributed to facilitated entry of the virus into cells. They suggested that other mechanisms could be responsible for this phenomenon.

The full-length CD4 molecule negatively regulates HIV-1 replication

To investigate the possibility that viral dissemination by cell-free virus and/or cell-to-cell contact might be more efficient in cells expressing t-CD4, the antiviral drug azidothymidine was added at a non-toxic inhibitory concentration (1 μM) to both cell lines 24 h after their initial contact with HIV particles. Addition of azidothymidine inhibits any further cycle of viral integration mediated either by cell-free virus or via cell-to-cell contact. However, it does not affect replication of viral DNA which is already integrated. Consequently, infected cells are derived only from the initial pool of infected cells. As expected, in the absence of drug, higher viral expression was observed in cells carrying t-CD4

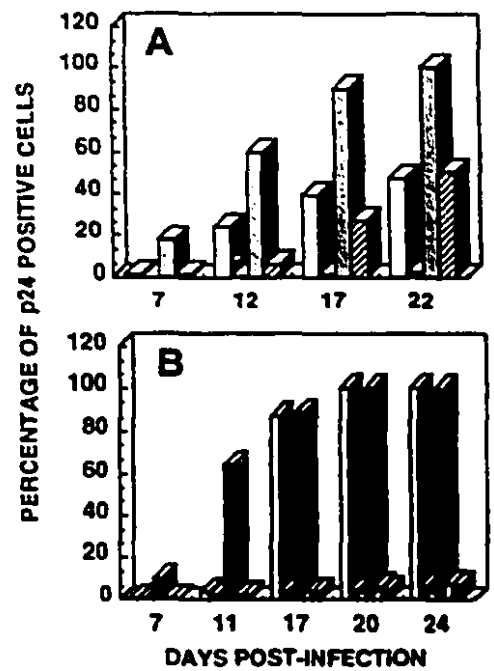

Fig. 5. Effect of azidothymidine and soluble CD4 on HIV-1 infection of wt-CD4 and t-CD4 A2.01 cells. (A) Cells (5×10^5) were infected with HIV-IIIB (m.o.i., 0.14) and were then treated with azidothymidine (1 μM) 24 h after exposure to HIV-1. Thereafter, the cells were kept under drug pressure. Viral infection was monitored by immunofluorescence to detect the main viral core protein p24. (□) untreated and (▨) azidothymidine-treated wt-CD4 A2.01 cells (A2D8); (□) untreated and (▨) azidothymidine-treated t-CD4 A2.01 cells (3D4D8). (B) Cells (5×10^5) were infected with HIV-IIIB (m.o.i., 0.5) prior to addition of sCD4 at 24 h post-infection. Viral replication was monitored by indirect immunofluorescence using an antibody specific for viral p24 protein. A2.01 cells carrying wt-CD4 (A2D8) either untreated (□), or treated with 2 (▨) or 20 μg/ml sCD4 (■), and A2.01 cells carrying t-CD4 (3D4D8) either untreated (■), or treated with 2 (▨) or 20 μg/ml sCD4 (■).

(100%) in comparison with cells transfected with wt-CD4 (47%) (Figure 5A). In azidothymidine-treated t-CD4 cells, the number of HIV-1-positive cells remains almost negative

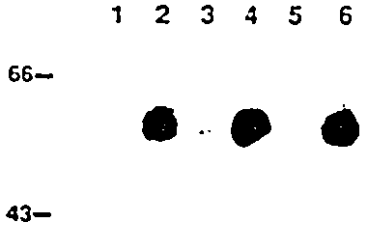

Fig. 6. An increase in autophosphorylation activity of the CD4-associated *lck* is induced by cross-linking of CD4. Cells (10^7) were incubated on ice with 1F3 anti-CD4 antibody at 5 μg/ml (lanes 2, 4 and 6) or with medium (lanes 1, 3 and 5) for 30 min, prior to cross-linking with a goat anti-mouse serum for 2 min at 37°C. CD4 was then immunoprecipitated and an *in vitro* kinase assay was performed. Proteins were resolved on a 7.5% SDS–polyacrylamide gel. Lanes 1 and 2, HSB-2 CD4 (A4); lanes 3 and 4, A2.01 A2D8; lanes 5 and 6, Jurkat cell line.

until ~12 days post-infection; however, the number of HIV-1 particles released into the medium (data not shown) was so important that despite the presence of azidothymidine, effective HIV-1 replication was observed over time with ~50% of cells expressing p24 22 days post-infection. Interestingly, viral replication was totally abrogated in cells expressing wt-CD4 even at the later time points (22 days post-infection). This experiment was repeated twice and yielded similar results. Results from these experiments suggest that viral replication is down-regulated in cells expressing wt-CD4 into which initial viral binding and internalization has occurred. This down-regulation is not observed in cells expressing t-CD4.

The negative signal is transduced via the CD4 molecule

To determine if the negative signal on viral replication was transduced by the CD4 molecule, different concentrations of soluble CD4 (sCD4) were added 24 h following HIV-1 infection of wt-CD4 and t-CD4 A2.01 cells. In these experiments, viruses which are already integrated are allowed to replicate. However, sCD4 will bind to viral particles released from infected cells, thereby preventing their interaction with wt-CD4 or t-CD4 on the surface of transfected cells. Results from these experiments are illustrated in Figure 5B. Addition of sCD4 at different concentrations (2 and 20 μg/ml) led to a significant decrease in the percentage of p24$^+$ cells in both wt-CD4- and t-CD4-expressing cells. The number of p24$^+$ cells ranged between 4.3 and 7% in cells incubated in the presence of 20 μg/ml sCD4 at 24 days post-infection, while all of the cells were infected in the absence of sCD4. Interestingly, in the presence of sCD4, we observed for the first time a similar percentage of p24$^+$ cells in both cell lines. In triplicate experiments with 20 μg/ml of sCD4, the percentage was ~4.3% in wt-CD4 cells and 7% in t-CD4 cells. Similar results were also observed when 2 μg/ml of sCD4 were added to these cultures (Figure 5B). These results strongly suggest that the CD4 molecule is responsible for transducing the signal which down-regulates HIV-1 replication.

The protein tyrosine kinase p56^{lck} plays a dominant role in the transduction of the negative signal

The CD4 molecule is associated in T cells with the *src* family tyrosine kinase p56^{lck} (Rudd *et al.*, 1988; Veillette *et al.*, 1988). Cross-linking of CD4 using anti-CD4 antibodies or gp120 leads to an increase in autophosphorylation activity of the CD4-associated *lck* (Veillette *et al.*, 1989; Koretzky *et al.*, 1990; Luo and Sefton, 1990; Hivroz *et al.*, 1993). It is thus possible that the above-described regulatory signal generated through CD4 involves p56^{lck}. We first verified if the CD4-associated *lck* could be activated in the HSB-2 CD4 A4 and the A2.10 A2D8 cell lines, following cross-linking with an anti-CD4 mAb that prevents gp120 binding to CD4. We observed a 3.2-fold (HSB-2 CD4 A4) and a 3.7-fold (A2.01 A2D8) enhancement in the autophosphorylation activity of the CD4-associated *lck* following a 2 min CD4 cross-linking at 37°C (Figure 6). This increase in *lck* autophosphorylation activity was comparable with the 3.6-fold enhancement observed upon CD4 cross-linking on Jurkat, which is a mature T cell line. Furthermore, a similar pattern of tyrosine phosphorylation of substrates was observed after cross-linking of CD4 at the surface of both Jurkat and A2.01 A2D8 cells (data not shown), suggesting that the CD4–*lck* association in HSB-2 CD4 and A2.01 A2D8 cell lines is functional.

To examine the involvement of the CD4-associated tyrosine kinase p56^{lck} in the above-described down-regulation of HIV-1 replication, a CD4 mutant lacking the residues responsible for p56^{lck} association was transfected into A2.01 and HSB-2 cells. Infection of A2.01 and HSB-2 cells expressing the C4202A mutant was carried out as described previously and compared with infection of wt-CD4

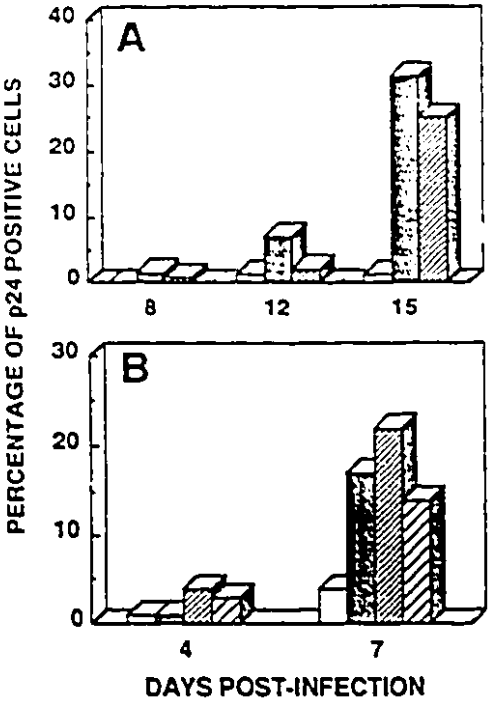

Fig. 7. HIV-1 infection of T cell lines transfected with the double cysteine mutant of CD4. (A) A2.01 cells (5×10^5) expressing either wt-CD4 (□), t-CD4 (□) or CD4 mutated at positions 420 and 422 (▨) were incubated with HIV-IIIB (m.o.i., 0.25) for 90 min at 37°C. Determination of cells expressing viral p24 protein was monitored by indirect immunofluorescence at 8, 12 and 15 days post-infection. (B) HSB-2 cells expressing either wt-CD4 (A1, □), t-CD4 (□) or CD4 mutated at positions 420 and 422 (clone 8, ▨; clone 17, ▨) were infected with HIV-1 (m.o.i., 0.03) and were processed as described above. Percentage of p24$^+$ cells was assessed at 4 and 7 days post-infection.

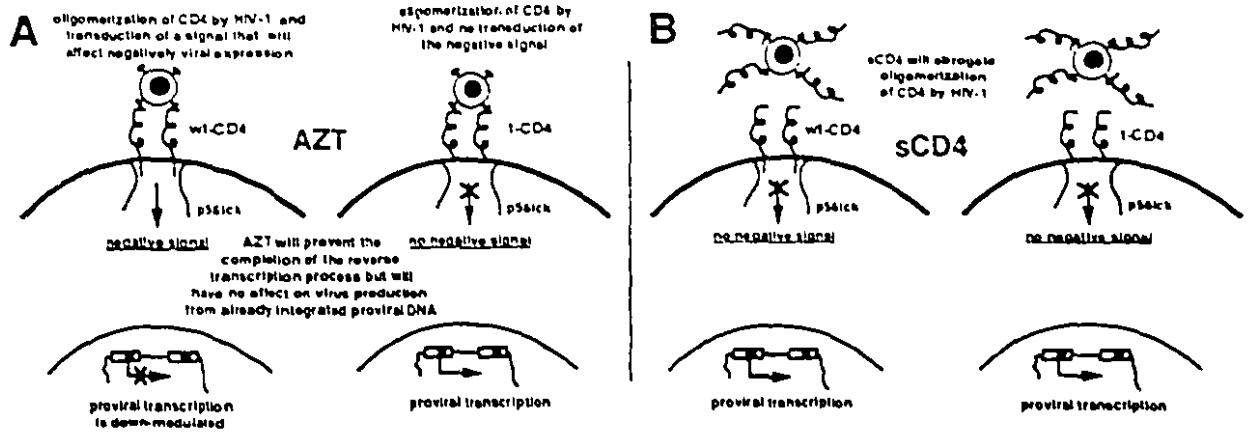

Fig. 8. Overview of the proposed cellular mechanisms that take place following binding of HIV-1 particles to cell surface CD4 based on studies with antiviral agents. In each case, infection with HIV-1 was performed prior to addition of antiviral agents. (A) Addition of azidothymidine will not inhibit virus replication from previously HIV-1-infected cells. Virions produced from these cells will bind to provirus-containing cells and, following cross-linking of CD4, will induce transduction of a signal that will negatively regulate the expression of viral DNA present in cells expressing wt-CD4 (left panel). This negative signal will not be transmitted in cells expressing t-CD4 due to the deletion of the cytoplasmic domain of CD4 which is responsible for the signaling function of the molecule (right panel). (B) Addition of sCD4 will abrogate the binding of virions secreted from previously HIV-1-infected cells. Consequently, the negative signal will not be transmitted since cross-linking of CD4 molecules will be prevented.

cells and t-CD4 cells. Results are illustrated in Figure 7A (A2.01) and B (HSB-2). A 6- to 20-fold increase in the percentage of $p24^+$ cells was detected in A2.01 (25%) and HSB-2 (21.6%) cells expressing the mutated form of CD4 when compared with A2.01 (1.25%) or HSB-2 (3.9%) cells transfected with the wt-CD4. This difference was observed throughout the different time points of the experiment. Furthermore, the percentage of $p24^+$ cells was almost identical in cells expressing the mutated form of CD4 and t-CD4 A2.01 (31.5%) and t-CD4 HSB-2 cells (16.6%). Results of these experiments indicate that the association of $p56^{lck}$ to CD4 contributes to the decreased viral replication observed in cells expressing the full-length form of CD4.

Discussion

We present evidence suggesting that the full-length CD4 molecule transduces a signal that will negatively regulate HIV-1 replication. Cells expressing a truncated form of CD4 or a mutant CD4 molecule which can no longer associate with $p56^{lck}$ permitted increased levels of viral production. These results were obtained using *independently* derived cell clones for each construct and two different strains of HIV-1 (HIV-IIIB and SF-2), thus eliminating the possibility of a strain-specific phenomenon. A very low m.o.i. was intentionally used in each experiment to parallel physiological conditions. Viral p24 protein levels and quantitative PCR were used to determine that the efficiency of viral entry and reverse transcription was similar following HIV-1 infection of cells expressing either wt-CD4 or t-CD4. Our results strongly suggest that the event responsible for the different levels of viral expression occurs following reverse transcription of viral RNA and prior to initiation of transcription.

The above-described experiments had indicated that viral entry was identical in cells expressing the different forms of CD4, suggesting that the quantitative differences observed in HIV-1 expression were due to later events in the viral replication cycle. Addition of azidothymidine to cells which had integrated comparable amounts of HIV-1 resulted in an enhancement of the differences observed in the percentage of $p24^+$ cells between t-CD4 cells and wt-CD4 cells. A 50-fold difference could be observed 23 days post-infection. These results can be explained by a model whereby viral particles released from cells infected prior to addition of azidothymidine bind to CD4 on such HIV-1-infected cells and induce multimerization of CD4. This leads to the transduction of a signal that negatively regulates the expression of already integrated viral DNA (Figure 8A). Alternatively, the negative signal could affect integration of viral DNA. This negative signal is not transmitted in cells carrying t-CD4 since deletion of the cytoplasmic domain of CD4 abrogates the interaction of CD4 with $p56^{lck}$ (Shaw *et al.*, 1990; Turner *et al.*, 1990), thus leading to enhanced viral replication. To confirm that CD4 was in fact playing a primordial role in the transduction of a putative negative signal, sCD4 was added to the cells soon after infection with HIV-1. This antiviral agent was used to abrogate multimerization of CD4 induced by HIV-1 particles released from cells into which viral integration had already occurred (Figure 8B). sCD4 also prevents *de novo* infection of $CD4^+$ cells mediated by cell–cell contact or cell-free virus. Results from this set of experiments demonstrated that the rate of virus replication was similar in cells carrying the wt-CD4 or t-CD4, again confirming that the initial infection was comparable in cells expressing both forms of CD4. These experiments clearly demonstrated that the negative regulatory effect results from the binding of HIV-1 to CD4. Most importantly, experiments using a mutated form of CD4 which is not associated with $p56^{lck}$ clearly demonstrated that this tyrosine kinase is implicated in the transduction of the negative signal (Figure 7).

Our results contrast with those of Poulin *et al.* (1991) who observed a delay of HIV-1 replication in A2.01 cells expressing a truncated version of CD4, lacking most of the cytoplasmic domain. These differences may be explained by the fact that they have used at least 100- to 1000-fold more virus particles for infection. Our results are consistent with a previous report which demonstrated that a glycolipid-anchored CD4 molecule, lacking membrane spanning and cytoplasmic domains, was associated with enhanced HIV-1 replication in the same human T-lymphoid cell line (HSB-2)

that has been used in our experiments (Diamond *et al.*, 1990). Two recent studies have also proposed that the CD4 glycoprotein could influence replication of HIV-1. Binding of recombinant HIV-like particles (containing only the envelope glycoprotein of HIV) to CD4 was shown to inhibit induction of latent HIV-1 (Haffar *et al.*, 1992). Interestingly, viral particles which contained another viral protein (*gag*) but not *env* did not mediate this effect on HIV-1 replication. Moreover, expression of increasing levels of CD4 in HSB-2 cells was shown to correlate with a marked decrease in HIV-1 replication, which is consistent with our results (Marshall *et al.*, 1992). In this report, similar levels of virus entry and reverse transcription were observed in cells expressing low and high levels of CD4, which is also similar to our data.

Results from our experiments suggest that binding of HIV-1 to cell surface CD4 stimulates signal transduction pathways in T cells. Recent evidence consistent with this scenario came from the observation that treatment of CD4-positive cells with gp120, followed by anti-gp120 antibodies, results in increased tyrosine kinase activity of p56^{lck} (Juszczak *et al.*, 1991). Our results also indicate that the signal leads to decreased viral replication. Cross-linking of CD4 molecules with CD4-specific mAbs or with gp120 prior to T cell receptor mediated stimulation has been shown to inhibit T cell activation and to induce T cell death (Newell *et al.*, 1990; Banda *et al.*, 1992). Several mechanisms including negative signaling by p56^{lck} (Juszczak *et al.*, 1991) or sequestration of p56^{lck} by CD4 (Haughn *et al.*, 1992) have been suggested to play a role in this down-regulation of T cell activation by CD4. T cell activation leads to enhanced HIV-1 replication indicating the presence of common pathways between these two responses. It is thus possible that signals which will negatively regulate T cell activation will also result in down-regulation of HIV-1 replication.

Numerous studies have demonstrated that various factors can affect negatively HIV expression at transcriptional or post-transcriptional levels. Transcriptional activity of HIV is regulated by virally encoded factors, such as *tat*, and also by inducible cellular transcription factors. Some of these factors (LP-1 and USF/MLTF) have been shown to regulate negatively HIV-1 transcription (Kato *et al.*, 1991; Giacca *et al.*, 1992). Another report has indicated that integrated HIV-1 genome is maintained in an inactive state by cellular factors binding to a region of the promoter located in the long terminal repeat between −120 and +80 relative to the transcription initiation site (Drysdale and Pavlakis, 1991). One particularly well-characterized nuclear transcription factor, NF-κB, has been reported to greatly stimulate the HIV-1 enhancer (Böhnlein *et al.*, 1988). An IκB-like molecule has been demonstrated to induce negative regulation of HIV-1 transcription by blocking the formation of the 65 kDa plus 50 kDa NF-κB heterodimer (Raziuddin *et al.*, 1991). Dissociation of NF-κB from its inhibitor IκB is achieved following phosphorylation of IκB by protein kinase C. It is thus possible that one of these factors is involved in the negative regulation of HIV-1 that we have observed in our experiments.

The *in vitro* studies presented here provide evidence that the cytoplasmic domain of CD4 can regulate the rate of HIV-1 expression in T cells. Our hypothesis is that, following binding of HIV-1 to CD4, a signal is transduced via p56^{lck} leading to phosphorylation of cellular and/or viral substrates and resulting in degradation of viral genome prior to integration or down-modulation of viral replication following efficient viral integration. The relevance of our observations to the *in vivo* situation is not known but it is tempting to speculate that it might play a role in the pathogenesis of this retroviral infection, particularly at the level of latency. The mechanism by which HIV remains latent in infected individuals is still unclear. It seems that most infected cells are transcriptionally silent since the frequency of cells expressing HIV-1 RNA has been shown to be one to two orders of magnitude lower than that of provirus-containing cells (Harper *et al.*, 1986; Psallidopoulos *et al.*, 1989; Schnittman *et al.*, 1989). Binding of cell-free virus present in the circulation (Coombs *et al.*, 1990; Ho *et al.*, 1990) to infected cells could lead to transcriptional down-regulation of HIV-1 integrants. The hypothesis that the great majority of proviruses in infected individuals are functionally defective is of interest in this regard (Goodenow *et al.*, 1989). Such defective particles, even though they could not initiate a full virus replicative cycle, might be able to multimerize the CD4 molecule, thus leading to the transduction of a signal that will negatively affect replication of integrated viral DNA and induce viral latency. This would prevent a quick destruction of the host and help maintain a sustained level of viral infection.

Materials and methods

Cells and media

A2.01 and HSB-2 are T-cell tumor cell lines which are CD4-negative and have been previously described (Folks *et al.*, 1986; Hara *et al.*, 1988). These cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 μM 2-mercaptoethanol and 1 mg/ml of geneticin (G-418) when required. Cell lines producing the recombinant retroviruses were maintained in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, 50 μM 2-mercaptoethanol and 1 mg/ml of G-418.

Expression of the full-length, truncated and mutated CD4 in A2.01 and HSB-2 cells

Wild-type and truncated CD4 coding regions were subcloned into the MNC retroviral vector containing a neomycin resistance gene (Peterson and Seed, 1988). The cytoplasmic region of CD4 was truncated by insertion of a stop codon in the coding sequence. Only the first six membrane-proximal residues were conserved. The generation of the double cysteine mutant was performed by overlap extension procedure. Briefly, DNA fragments containing the mutation were amplified using 250 ng of MNC plasmid containing the wild-type CD4 cDNA as template, 100 ng of either of complementary primers containing the desired substitutions (5'-CCGGTGAGGGGCCTGGGCGGT-CTTCTT-3' or 5'-AAGAAGACCGCCCAGGCCCCTCACCGG-3'), 100 ng of 5' or 3' primers hybridizing to MNC vector sequences and a reaction mix containing 2.5 U of Taq polymerase, 0.2 mM of each of the dNTPs, 100 mM KCl, 10 mM Tris–HCl pH 9.0, 1.5 mM $MgCl_2$, 0.01% gelatin and 0.1% Triton X-100. The DNA was amplified for 20 cycles consisting of denaturation for 30 s at 94°C, annealing for 2 min at 50°C and elongation at 72°C for 2 min, followed by an elongation cycle of 15 min at 72°C. The mutated cDNA was generated in a subsequent overlap-extension amplification using 250 ng of each of the two overlapping fragments, 100 ng of each of the vector encoded 5' and 3' primers and the same amplification conditions as above. The full-length mutated cDNA was then cloned in the *Hin*dIII–*Bam*HI sites of the eukaryotic expression vector MNC stuffer. The whole cDNA was sequenced using Sanger's method to confirm the presence of the desired substitutions and the integrity of the rest of the cDNA. The amphotropic helper packaging cell line DAMP (Sleckman *et al.*, 1988) was transfected by calcium phosphate co-precipitation with the different pMNC constructs and G-418 resistant DAMP cells were selected and used as producer cell lines of the recombinant retrovirus. DAMP cells transfected with the different pMNC-CD4 constructs were enriched for cells expressing high levels of membrane CD4 by cell sorting. For infection, DAMP cells, at 70% confluence, were treated with 10 μg/ml

of mitomycin C for 3 h at 37°C and washed extensively with phosphate buffered saline (PBS) prior to co-culture with A2.01 and HSB-2 cells (1×10^6) in complete RPMI medium in the presence of 8 μg/ml of polybrene for 24 h at 37°C. Cells were washed three times with PBS and resuspended in complete RPMI medium for 24 h at 37°C. The cells were finally seeded at a density of 50 000 cells per well (2 ml) in complete RPMI medium containing 1 mg/ml of G-418. Medium was changed twice a week and G-418 resistant A2.01 and HSB-2 cells were analyzed for the expression of CD4 molecules by flow cytometry.

Flow cytometry analysis

Cells were incubated with 1F3 anti-CD4 monoclonal antibody at 5 μg/ml (a gift from A.Truneh, SmithKline Beecham Pharmaceuticals, King of Prussia, PA, USA) for 30 min at 4°C, washed with PBS and incubated under similar conditions with FITC-coupled goat anti-mouse IgG at 2 μg/ml (Southern Biotechnology Associates, Inc., Birmingham, AL, USA). Cells were washed once and resuspended in PBS prior to analysis using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA).

Viruses

Viral strains used in this study were HIV-IIIB (Gallo *et al.*, 1984) and SF-2 (Cheng-Mayer and Levy, 1988) harvested from culture fluids of chronically infected H-9 and HUT-78 T-lymphoid cell lines, respectively. Titers of these HIV-1 stocks and evaluation of progeny virus production were determined by end-point titration using highly susceptible MT-4 cells as targets (Harada *et al.*, 1985). Four parallel series of 10^6 fold dilutions were used. Cells from each well were stained 7–10 days post-infection by indirect immunofluorescence (see below) and $TCID_{50}$ was calculated according to Reed and Muench (1938). We have estimated that, as previously reported (Tjotta *et al.*, 1990), one $TCID_{50}$ unit is equal to a single infectious viral particle. Viral infection studies were performed by exposing studied cells to HIV-1 at 37°C for 2.5 h. Cells were then washed twice with medium and resuspended in fresh medium. In some experiments, azidothymidine or soluble CD4 was included 24 h post-viral infection. Evaluation of viral entry was determined following incubation of 5×10^5 cells with HIV-1 (m.o.i., 1.0) on ice for 30 min to permit viral binding. Incubation was then performed at 37°C for 90 min to allow viral penetration and cells were washed three times with cold PBS. Cells were finally lysed with Triton X-100 (1%) in PBS and assayed by an ELISA test for p24 protein according to manufacturer's instructions.

Immunofluorescence and measurement of p24 protein

Specific major viral core protein (p24) was detected by an indirect immunofluorescence assay. Mouse mAbs specific for p24 (kindly supplied by Dr R.C.Gallo) were used in conjunction with a FITC-conjugated goat anti-mouse IgG (BRL, Gaithersburg, MD, USA). At least 200 cells were counted in order to evaluate the percentage of p24-expressing cells. Cells infected with HIV-1 were quantitatively assayed for levels of p24 protein in culture supernatants using a commercial enzyme immunoassay according to manufacturer's specifications (Abbott Laboratories, Chicago, IL, USA).

Reverse transcriptase assay

Poly(rA)-dependent DNA polymerase activity was measured by a modification of a previously reported procedure (Hoffman *et al.*, 1985). In brief, cell-free supernatants were ultracentrifuged at 100 000 r.p.m. for 30 min at 4°C and pellets were resuspended in 10 μl of serum-free RPMI-1640. Samples (10 μl) were incubated with 50 μl of a buffer containing 50 mM Tris–HCl; 5 mM $MgCl_2$; 150 mM KCl; 0.05% Triton X-100; 0.3 mM GSH; 0.5 mM EGTA; 50 μg/ml poly(rA)p$(dT)_{12-15}$; 10 μCi thymidine triphosphate (40–70 Ci/mM) and 17.25 μl distilled water for 1 h at 37°C. The reaction was stopped by placing the tubes on ice and by adding 2 ml of sodium pyrophosphate 0.01 M (in 1 M HCl) and 2 ml of ice-cold 10% trichloroacetic acid (TCA). Precipitates were collected on Whatman GF/C fiber filters, which were washed several times with ice-cold 5% TCA, rinsed once with 70% ethanol and air-dried, prior to counting in a liquid scintillation spectrometer.

Quantitative PCR analysis

Cellular DNA used for amplification was prepared as follows. Cells (1.5×10^5) were washed in PBS and the cell pellet was treated with proteinase K (120 μg/ml) for 1 h at 60°C in 75 μl lysis buffer (1:1, 100 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$: 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 1% Tween-20, 1% NP-40). Proteinase K was then inactivated by boiling at 95°C for 1 h. Samples were cooled to room temperature and stored at −85°C (until assayed). The PCR reaction mixture contained 1 μg of DNA, 20 pmol of unlabeled primers, 20 pmol of ^{32}P-labeled primer, 250 μM each of four deoxynucleotide triphosphates, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl and 2.5 U of Taq DNA polymerase (Perkin–Elmer Cetus). This reaction mixture was covered with 25 μl of mineral oil and then subjected to denaturation (1 min at 94°C), and extension at 65°C for 30 cycles. Products from PCR amplifications were resolved on a non-denaturing 6% polyacrylamide gel and visualized by direct autoradiography of the gel. Primer pairs used in these experiments included M667/M661 which detects the primer binding site of HIV-1 and a pair of oligonucleotide primers (14-33/123-104) that amplify a region located in the first exon of the human β-globin gene (Zack *et al.*, 1990). A 200 bp fragment is amplified using M667/M661, while a 100 bp fragment is obtained when using 14-33/123-104.

Western blot analysis

Cellular lysates were prepared and subjected to electrophoresis through 10% polyacrylamide gels. The resolved proteins were then transferred onto nitrocellulose filters. Filters were saturated with a 5% milk solution and incubated overnight at 4°C with 1:500 v/v dilution of polyclonal sera from HIV-1-infected subjects. Filters were washed several times and incubated for 60 min with ^{125}I-labeled recombinant protein A/G (Calbiochem, La Jolla, CA, USA) (0.5×10^6 c.p.m./ml), washed, dried and exposed to X-ray films.

Northern blot analysis of HIV-1 RNA

Total cellular RNA was isolated at specific time points following viral infection by a modified guanidium isothiocyanate procedure (Chomczynski and Sacchi, 1987). Similar amounts of total cellular RNA (10 μg) were resolved on formaldehyde–1.0% agarose gels and transferred to GeneScreenPlus membrane. Filters were hybridized with random-primed ^{32}P-labeled products of pBH-10 (Shaw *et al.*, 1984) and of mouse actin (Minty *et al.*, 1981) to detect HIV-1 and actin, respectively. Filters were washed in 2 × SSC–0.1% SDS followed by 0.1 × SSC–0.1% SDS at 64°C. Blots were exposed to X-OMAT film (Kodak) at −70°C with intensifying screens for 24 h.

[^{125}I]rgp120 binding assay

Soluble immunopurified recombinant gp120 (rgp120), kindly provided by Genentech (Dr T.Gregory), was radioiodinated by the lactoperoxidase method (Marchalonis, 1969). Cells were incubated with increasing concentrations of [^{125}I]rgp120 for 4 h at 25°C with gentle mixing in a final volume of 200 μl RPMI-1640, 25 mM HEPES and 0.1% BSA (pH 7.4). Non-specific binding was evaluated by using a 100-fold excess of unlabeled rgp120. Cells were then layered onto a 200 μl oil cushion made of 84% silicone oil and 16% paraffin oil and were centrifuged at 17 000 *g* for 2 min. Cell pellets were obtained by cutting off the tip of the tube and cell-bound [^{125}I]rgp120 radioactivity was measured in a gamma counter. Average of duplicate determinations of bound [^{125}I]rgp120 were used for data analysis. Saturation binding curves were analyzed by non-linear least-squares curve fitting according to mass action law (DeLéan *et al.*, 1982).

Autophosphorylation assay

An increase in autophosphorylation activity of the CD4-associated *lck* was observed following the cross-linking of CD4. Briefly, 10^7 cells were incubated with anti-CD4 antibody 1F3 at 5 μg/ml or with stimulation medium (DMEM, BSA 0.5%, HEPES 10 mM) for 30 min on ice. Cells were then washed, treated with a goat-anti-mouse serum (Sigma) at 50 μg/ml for 2 min at 37°C, and then lysed in NP-40 lysis buffer (NP-40 2%, DOC 0.5%, Tris 50 mM, NaCl 150 mM, NaF 50 mM, $NaPO_7$ 10 mM, EGTA 2 mM, EDTA 10 mM, Na orthovanadate 500 μM, leupeptin 3×10^{-6} M, pepstatin A 2×10^{-6} M and PNGB 50 μM). CD4 was then immunoprecipitated with protein A–Sepharose precoated with a rabbit anti-CD4 serum. Autophosphorylation was performed as follows. After extensive washes, the immunoprecipitates were resuspended in 50 μl of kinase buffer (10 μCi of [γ-^{32}P]ATP, 50 μM of cold ATP, Na orthovanadate 100 μM, PNGB 50 μM, $MgCl_2$ 20 mM, $MnCl_2$ 10 mM, Triton X-100 0.1% and HEPES 20 mM) for 10 min at room temperature. The assay was stopped with 1 ml of ice-cold buffer (HEPES 20 mM, Triton X-100 0.1%, Na orthovanadate 100 μM, PNGB 50 μM) and washed three times before addition of sample buffer. Proteins were then resolved on a 7.5% SDS–polyacrylamide gel. The gel was then dried and exposed to an X-ray film for 2 h. The gel was also exposed to Kodak storage phosphor screens and quantitation was done on PhosphorImager devised by the ImageQuant Software Package (Molecular Dynamics, Sunnyvale, CA, USA).

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References

Abraham,N., Miceli,M.C., Parnes,J.R. and Veillette,A. (1991) *Nature*, 350, 62–66.

Arthos,J., Deen,C.K., Chaikin,M.A., Fornwald,J.A., Sattentau,Q.J., Clapham,P.R., Weiss,R.A., McDougal,J.S., Pietropaolo,C., Maddon,P.J., Truneh,A., Axel,R. and Sweet,R.A. (1989) *Cell*, 57, 469–481.

Banda,N.K., Bernier,J., Kurahara,D.K., Kurrle,R., Haigwood,N., Sékaly,R.-P. and Helman Finkel,T. (1992) *J. Exp. Med.*, 176, 1099–1106.

Bank,I. and Chess,L. (1985) *J. Exp. Med.*, 162, 1294–1303.

Bedinger,P., Moriarty,A., von Borstel,R.C., Donovan,N.J., Steiner,K.S. and Littman,D.R. (1988) *Nature*, 334, 162–165.

Böhnlein,E., Lowenthal,J.W., Siekevitz,M., Ballard,D.W., Franza,B.R. and Green,W.C. (1988) *Cell*, 53, 827–836.

Cheng-Mayer,C. and Levy,J.A. (1988) *Anal. Neurol.*, 23, 558–561.

Chomczynski,P. and Sacchi,N. (1987) *Anal. Biochem.*, 162, 150–159.

Coombs,R.W., Collier,A.C., Allain,J.P., Nikora,B., Leuther,M. and Gjerset,G.F. (1989) *N. Engl. J. Med.*, 321, 1626–1631.

DeLéan,A., Hancock,A.A. and Lefkowitz,R.J. (1982) *Mol. Pharmacol.*, 21, 5–16.

Diamond,D.C., Finberg,R., Chauhuri,S., Sleckman,B.P. and Burakoff,S.J. (1990) *Proc. Natl Acad. Sci. USA*, 87, 5001–5005.

Drysdale,C.M. and Pavlakis,G.N. (1991) *J. Virol.*, 65, 3044–3051.

Fleury,S., Lamarre,D., Meloche,S., Ryu,S.-E., Cantin,C., Hendrickson,W.A. and Sékaly,R.-P. (1991) *Cell*, 66, 1037–1049.

Folks,T., Powell,D.M., Lightfoote,M.M., Benn,S., Martin,M.A. and Fauci,A.S. (1986) *Science*, 231, 600–602.

Gallo,R.C., Salahuddin,S.Z., Popovic,M., Shearer,G.M., Kaplan,M., Haynes,B.F., Palker,T.J., Redfield,R., Oleske,J., Safai,G., White,G., Foster,P. and Markham,P.D. (1984) *Science*, 224, 500–503.

Gay,D., Maddon,P., Sékaly,R.-P., Talle,M.A., Godfrey,M., Long,E., Goldstein,G., Chess,L., Axel,R., Kappler,J. and Marrack,P. (1987) *Nature*, 328, 626–629.

Geppert,T.D. and Lipsky,P.E. (1987) *J. Immunol.*, 138, 1660–1666.

Giacca,M., Gutierrez,M.I., Menzo,S., D'adda Di Fagagna,F. and Falaschi,A. (1992) *Virology*, 186, 133–147.

Glaichenhaus,N., Shastri,N., Littman,D.R. and Turner,J.M. (1991) *Cell*, 64, 511–520.

Goodenow,M., Huet,T., Saurin,W., Kwok,S., Sninsky,J. and Wain-Hobson,S. (1989) *J. Acq. Immun. Def. Synd.*, 2, 344–352.

Haffar,O.K., Moran,P.A., Smithgall,M.D., Diegel,M.L., Sridhar,P., Ledbetter,J.A., Zarling,J.M. and Hu,S.L. (1992) *J. Virol.*, 66, 4279–4287.

Hara,J., Benedict,S.H., Champagne,E., Mak,T.W., Minden,M. and Gelfand,E.W. (1988) *J. Clin. Invest.*, 81, 989–996.

Harada,S., Koyanagi,Y. and Yamamoto,N. (1985) *Science*, 229, 563–566.

Harper,M.E., Marselle,L.M., Gallo,R.C. and Wong-Staal,F. (1986) *Proc. Natl Acad. Sci. USA*, 83, 772–776.

Haughn,L., Gratton,S., Caron,L., Sékaly,R.-P., Veillette,A. and Julius,M. (1992) *Nature*, 358, 328–331.

Hivroz,C., Mazerolles,F., Soula,M., Fagard,R., Gratton,S., Meloche,S., Sékaly,R.-P. and Fischer,A. (1993) *Eur. J. Immunol.*, 23, 600–607.

Ho,D.D., Tarsem Moudgil,M.S. and Masud Alam,M.S. (1990) *N. Engl. J. Med.*, 321, 1621–1625.

Hoffman,A.D., Banapour,B. and Levy,J.A. (1985) *Virology*, 147, 326–335.

Juszczak,R.J., Turchin,H., Truneh,A., Culp,J. and Kassis,S. (1991) *J. Biol. Chem.*, 266, 11176–11183.

Kato,H., Horikoshi,H. and Roeder,R.G. (1991) *Science*, 251, 1476–1479.

Korezsky,G.A., Picus,J., Schultz,T. and Weiss,A. (1990) *Proc. Natl Acad. Sci. USA*, 88, 2037–2041.

Lamarre,D., Ashkenazi,A., Fleury,S., Smith,D.H., Sékaly,R.-P. and Capon,D.J. (1989) *Science*, 245, 743–746.

Landau,N.R., Warton,M. and Littman,D. (1988) *Nature*, 334, 159–162.

Lori,F., Veronese,F.M., De Vico,A.L., Lusso,P., Ratz,M.S. and Gallo,R.C. (1992) *J. Virol.*, 66, 5067–5074.

Luo,K and Sefton,B.M. (1990) *Mol. Cell. Biol.*, 10, 5305–5313.

Maddon,P.J., McDougal,J.S., Clapham,P.R., Dalgleish,A.G., Jamal,S., Weiss,R.A. and Axel,R. (1988) *Cell*, 54, 865–874.

Marchalonis,J.J. (1969) *Biochem. J.*, 113, 299–305.

Marshall,W.L., Diamond,D.C., Kowalski,M.M. and Finberg,R.W. (1992) *J. Virol.*, 66, 5492–5499.

Minty,A.J., Caravatti,M., Robert,B., Cohen,A., Daubas,P., Weydert,A., Gras,F. and Buckingham,M.E. (1981) *J. Biol. Chem.*, 256, 1008–1014.

Mizukami,T., Fuerst,T.R., Berger,E.A. and Moss,B. (1988) *Proc. Natl Acad. Sci. USA*, 85, 9273–9277.

Molina,T.J., Kishihara,K., Siderovski,D.P., van Ewijk,W., Narendran,A., Timms,E., Wakeham,A., Paige,C.J., Hartman,K.-U., Veillette,A., Davidson,D. and Mak,T.W. (1992) *Nature*, 357, 161–164.

Newell,M.K., Haughn,L.J., Maroun,C.R. and Julius,M.H. (1990) *Nature*, 347, 286–289.

Peterson,A. and Seed,B. (1988) *Cell*, 54, 65–72.

Poulin,L., Evans,L.E., Tang,S., Barboza,A., Legg,H., Littman,D.R. and Levy,J.A. (1991) *J. Virol.*, 65, 4893–4901.

Psallidopoulos,M.C., Schnittman,S.M., Thompson,L.M., Baseler,M., Fauci,A.S., Lane,H.C. and Salzman,N.P. (1989) *J. Virol.*, 63, 4626–4631.

Raziuddin, Mikovits,J.A., Calvert,I., Ghosh,S., Kung,H.F. and Ruscetti,F.W. (1991) *Proc. Natl Acad. Sci. USA*, 88, 9426–9430.

Reed,L.J. and Muench,H. (1938) *Am. J. Hyg.*, 27, 493–497.

Rudd,C.E., Trevyllyan,J.M., Dasgupta,J.D., Wong,L.L. and Schlossman,S.F. (1988) *Proc. Natl Acad. Sci. USA*, 85, 5190–5194.

Ryu,S.E., Kwong,P.D., Truneh,A., Porter,T.G., Arthos,J., Rosenberg,M., Dai,H., Xuong,N.-h., Axel,R., Sweet,R.W. and Hendrickson,W.A. (1990) *Nature*, 348, 419–426.

Sattentau,Q. and Weiss,R.A. (1988) *Cell*, 52, 631–633.

Schnittman,S.M., Psallidopoulos,M.C., Lane,H.C., Thompson,L., Baseler,M., Massari,F., Fox,C.H., Salzman,N.P. and Fauci,A.S. (1989) *Science*, 245, 305–308.

Shaw,G.M., Hahn,B.H., Arya,S.K., Groopman,J.E., Gallo,R.C. and Wong-Staal,F. (1984) *Science*, 226, 1165–1171.

Shaw,A.S., Chalupny,J., Whitney,J.A., Hammond,C., Amrein,K.E., Kavathas,P., Sefton,B.M. and Rose,J.K. (1990) *Mol. Cell. Biol.*, 10, 1853–1862.

Sleckman,B.P., Peterson,A., Foran,J.A., Gorga,J.C., Kara,C.J., Strominger,J.L., Burakoff,S.J. and Greenstein,J.L. (1988) *J. Immunol.*, 141, 49–54.

Swain,S.L. (1983) *Immunol. Rev.*, 74, 129–142.

Tjotta,E., Hungnes,O. and Grinde,B. (1990) *J. Virol. Methods*, 27, 169–174.

Trono,D. (1992) *J. Virol.*, 66, 4893–4900.

Turner,J.M., Brodsky,M.H., Irving,B.A., Levin,S.D., Perlmutter,R.M. and Littman,D.R. (1990) *Cell*, 60, 755–765.

Veillette,A., Bookman,M.A., Horak,E.M. and Bolen,J.B. (1988) *Cell*, 55, 301–308.

Veillette,A., Bookman,M.A., Horak,E.M., Samelson,L.E. and Bolen,J.B. (1989) *Nature*, 338, 257–259.

Wang,J. Yan,Y., Garrett,T.P.J., Liu,J., Rodgers,D.W., Garlick,R.L., Tarr,G.E., Husasain,Y., Reiherz,E.L. and Harrison,S.C. (1990) *Nature*, 348, 411–428.

Wilde,D.B., Marrack,P., Kappler,J., Dialynas,D.P. and Fitch,F.W. (1983) *J. Immunol.*, 131, 2178–2183.

Zack,J.A., Arrigo,S.J., Weitsman,S.R., Go,A.S., Haislip,A. and Chen,I.S.Y. (1990) *Cell*, 61, 213–222.

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CHAPTER 6

HIV-1 NEF DOWNREGULATES CD4 EXPRESSION BY A BI-MODAL MECHANISM

I. Preface

The HIV Nef protein was demonstrated to down regulate cell surface expression of CD4. Moreover, Nef can upregulate HIV replication *in vitro*. In the last chapter, we have demonstrated that the CD4/lck association could negatively modulate HIV replication. An interesting hypothesis is that down regulation of cell surface CD4 would inhibit the negative signal generated through CD4/lck and thus contribute to the positive effect of Nef on HIV replication. We first characterized in this chapter the mechanisms by which Nef down regulates CD4 expression by using the vaccinia expression vector to transiently express Nef and CD4 in a CD4 negative human T cell line.

2. ABSTRACT

The mechanisms of HIV-1 Nef induced downregulation of CD4 were investigated using recombinant vaccinia virus systems to deliver Nef transiently to human T cell lines expressing CD4 or CD4 mutants. We show that Nef reduced the cell surface CD4 by promoting receptor endocytosis and lysosomal degradation. Amino-acid substitutions at the protein kinase-C (PKC) sensitive serines in the CD4 cytoplasmic tail, and changes at the two cysteines of the -CCQC- sequence that abolished the p56^{lck} kinase (*lck*) interaction preserved the Nef response. However, a CD4 mutant in which a -L-L- sequence in the cytoplasmic tail upstream of the *lck* interaction motif was substituted for two alanines, was not down-regulated by Nef or PMA. Nef failed to modulate the turn-over of other cell surface proteins containing di-leucine motif(s) suggesting that other CD4 sequences are required. In contrast to the enhanced endocytosis of CD4 receptor induced by Nef in the CD4 expressing cell lines, co-expression of Nef and CD4 in CD4 negative T cells led to a biphasic defect in the synthesis and surface expression of CD4. There was a quantitative defect early in CD4 biosynthesis that was followed by further degradation of CD4 due to the enhanced turnover of the receptor at the cell surface. The extracellular domain of CD4 was mapped as the target for the Nef effect on biosynthesis. The bimodal effect of Nef implies recruitment of cellular factors in the CD4 degradative pathway(s) and may have profound significance in T cell activation and HIV pathogenesis.

3. INTRODUCTION

Among the accessory proteins encoded by HIV-1, the 27 KDa myristoylated Nef protein (5, 28, 29) is dispensable for viral replication *in vitro*. Nef proteins of HIV-1 isolates exhibit significant divergence at the primary structure level and in some isolates the Nef protein is prematurely terminated (18). Of the three HIV encoded early proteins (Tat, Rev and Nef) that are expressed from multiply spliced mRNAs early in infection (55, 77), Nef has remained elusive to definition of its exact role(s) in HIV life-cycle. Although several earlier reports ascribed a negative regulatory effect of Nef on HIV replication and transcription (3, 50, 58, 59, 83), later studies have questioned this interpretation (36, 41) and more recently Nef has been presumed to be a positive regulator of HIV replication (17, 53, 82). In contrast to these *in vitro* tissue culture studies, animal pathogenesis studies have shown that Nef expression was crucial to the maintenance of high virus load and disease progression during natural infection (40). Nef has also been implicated in the regulation of cellular gene expression, such as down-regulation of the CD4 and IL-2 receptor (34) , IL-2 (51), and enhancer binding proteins, NFκb and AP-1 (56, 57). Of these phenomena, only the effect on CD4 is widely agreed upon.

The CD4 receptor in T cells is a major determinant of T cell activation (52, 72, 91). In resting T cells, CD4 is tightly associated with the $p56^{lck}$ tyrosine kinase (7, 73, 78, 85, 89). In T helper cells, antigen stimulation leads to interaction of CD4 with the T cell receptor and MHC class II receptor (21) thus favoring enhanced T cell activation. CD4 is also phosphorylated on serine residue(s) in its cytoplasmic tail following protein kinase C (PKC) activation by mitogen treatment or by antibody cross-linking of the T-cell receptor; this results in dissociation of $p56^{lck}$ and CD4 endocytosis (1, 37, 90, 92).

Since the original report by Guy et al (35), who demonstrated a loss of cell surface CD4 in CEM cells infected with a Nef expressing vaccinia virus, other groups have confirmed this property for both HIV and SIV Nef(6, 11, 30-32, 34). Using $CD4^+$ T cell lines that had been stably transduced with retroviral vectors expressing Nef, CD4 down-regulation was shown to occur post-translationally and to be independent of protein kinase C mediated phosphorylation of serine in the intracellular domain of CD4 (32). Subsequently, the Nef effect on CD4 was shown not to be a species-specific phenomenon and not to require human-specific factors. Moreover, it is not cell type specific, suggesting that its effect on CD4 was an intrinsic property of Nef (30). In vivo

studies with transgenic mice expressing HIV-1 Nef showed retarded T-cell ontogeny and severe depletion of peripheral blood CD4 $^{+}$ T-cells (12, 48, 81). More recently, it was shown that Nef mediates an endocytotic degradation of CD4 and the cytoplasmic domain of CD4 was necessary and sufficient for these effects (4, 6, 71). Importantly, a unique di-leucine motif in the cytoplasmic domain is critical for the Nef mediated CD4 endocytosis (4).

The objectives of this study were 1) to clarify a role for the CD4 cytoplasmic tail in the receptor endocytotic process induced by Nef and 2) to inquire whether the endocytotic process is the sole mechanism of CD4 down-modulation. Using the recombinant vaccinia virus system, we have compared the effects of acute Nef expression on both the pre-synthesized and co-expressed CD4 molecule, and demonstrate that Nef downregulates CD4 expression through a bi-modal mechanism. We confirm that the Nef induced CD4 endocytosis requires a CD4 specific membrane proximal sequence containing a -L-L- motif (4). When Nef and CD4 were co-expressed by simultaneous infection of T cells with the respective vaccinia recombinants, there was a quantitative defect in the biosynthetic rates of CD4 that was followed by enhanced turnover of the nascent receptor at the cell surface. The extracellular domain of CD4 was mapped as the target for the Nef effect on biosynthesis.

4. EXPERIMENTAL PROCEDURES

Cells and Viruses

Human T cell lines expressing wild type or mutant CD4 or CD4 chimera were constructed by retroviral transduction of the CD4 negative T cell line A2.01 using the MNC retroviral vector and G-418 selection (68, 84) (Gratton et al, unpublished) and were maintained in RPMI medium supplemented with 10% fetal bovine serum and G418 (500 µg/ml). The structures of the various mutants and chimeras are described in the relevant areas of the text. Rat basophilic leukemia cell lines that constitutively express the IL2-receptor/CD3 gamma chain chimera or the IL-2R/DKQTLL chimera were generously provided by Juan Bonifacino and Richard Klausner of NICHD.

All the recombinant vaccinia viruses used in this work were constructed by homologous recombination at the TK locus of the vaccinia virus genome. Plasmid pSC11 was used as the transfer vector. pSC11 contains the vaccinia virus 7.5K promoter for the expression of foreign genes and also had *E.coli* lac-Z gene linked to the vaccinia virus 11k promoter. The following recombinant vaccinia viruses were used: 1) for negative controls, either the parental WR strain or the recombinant Vaccinia virus (vSC8) encoding the *E.coli* lac-Z (15); 2) vvNef that was isogenic with vSC8 except for the Nef gene linked to the 7.5K promoter (43); 3) vvCD4 (vCB-7) expressing wt full-length human CD4 (14); 4) vvtCD4 (vCB-2) expressing CD4 truncated at residue 402 and lacking the cytoplasmic domain (Broder, C , unpublished data); 5) vvsCD4 (vCB5), expressing the secreted form of CD4, truncated at residue 375, and lacking both the trans-membrane (TM) and the cytoplasmic domains (13); 6) vvgp160 (vPE16) expressing the HIV-1 envelope glycoprotein, gp160 (23-25); 7) vvgp120 (vPE8) expressing the large subunit, gp120 of the *env* glycoprotein (23-25); and 8) vvCD46, expressing the human CD46 protein (60). All experiments utilized purified virus. Cells were infected with an m.o.i of 5 pfu/cell unless indicated otherwise.

Antibody binding and Flow Cytometric analysis

For determination of cell surface antibody binding, 10^5 cells from the respective infections were collected by centrifugation and washed with PBS. They were then incubated for 30-60 min at 4°C with the respective flurochrome conjugated or unconjugated monoclonal or rabbit polyclonal antibodies in 0.1 ml of PBS containing 3% BSA or 2% fetal calf serum (FCS) and 0.02% sodium azide. The cells were washed once with ice cold PBS containing BSA or FCS , then resuspended in 200 ml of PBS with BSA or FCS and incubated with fluorochrome conjugated secondary antibodies for 30

min at 4°C. The cells were then washed three times with ice cold PBS and fixed in PBS containing 4% paraformaldehyde. For detection of internal antigens, the cells were permeabilized with 0.1% saponin treatment and fixed in 4% paraformaldehyde prior to antibody binding. Flow cytometric data acquisition and analysis was performed using the Becton Dickinson FACSort^{TM} flow cytometer and LYSYS-II^{TM} or CELLQUEST^{TM} software.

Immunoblotting

Cells were harvested 4 hr after infection and one third equivalent from each dish collected by centrifugation. The cells were washed with PBS, then lysed by freeze-thawing in 200 ml of lysis buffer (50mM Tris-HCl, pH 8.0, 5mM EDTA, 100mM NaCl, 0.5% CHAPS, 0.5% Triton X-100), nuclei and debris were removed by brief centrifugation and aliquots separated on 12% SDS polycarylamide gels. After gel electrophoretic separation, the proteins were blotted to 0.2 micron Immobilon filters (Millipore). The membranes were reacted with polyclonal rabbit antisera against CD4 and Nef or monoclonal antibody against CD8, followed by reaction with the appropriate secondary antibody conjugated with horseradish peroxidase and the proteins were visualized by autoradiography following development with a chemiluminescence substrate (Amersham Corp).

Metabolic labeling and immunoprecipitation

For metabolic labeling experiments, CD4 $^{-}$ A2.01 or CD4 $^{+}$ SupT1 cells were routinely used. T cell lines infected with recombinant vaccinia virus (as described in the appropriate figure legends) were harvested between 2-4 hr after virus adsorption and incubated in methionine and cysteine free RPMI (containing 2% dialyzed FCS) for 10 min. For measuring the kinetics of protein biosynthesis, 10^7 cells were labeled for the indicated periods in 500 µl of methionine and cysteine free RPMI containing 2mCi/ml [^{35}S] Trans-label (ICN Corp). Equal aliquots were removed at 1, 2, 5 and 10 min and diluted in 20 volumes of ice cold PBS. The cells were collected by centrifugation in a microfuge for 10 sec, washed twice in PBS and processed for SDS/PAGE analysis. For pulse-chase experiments 2 X 10^7 cells were pulse-labeled for 10-30 min (or as specified in text) in 500 µl methionine and cysteine free RPMI containing [^{35}S] Trans-label. At the end of the pulse, the cells were diluted with 10 volumes of complete RPMI medium. Aliquots were removed immediately after labeling and at the indicated periods during the chase (from 0 to 14 hr). The cells were then collected by centrifugation and were disrupted by three cycles of freezing thawing in 500 µl of lysis buffer containing 0.05 M

TRIS-HCl, pH 7.4, 0.25% NP40 (or CHAPS), 0.25% Triton-X100, PMSF (10 mM), leupeptin (2 mM), pepstatin (3 mM), and aprotinin (2.5 mg/ml). The lysates were cleared by centrifugation prior to immunoprecipitation. In experiments using brefeldin A or lysosomal inhibitors, the cells were incubated in fresh medium containing the appropriate drugs (brefeldin A at 2 mg/ml, chloroquine at 100 mM, or ammonium chloride at 75 mM) for 1hr prior to pulse/chase labeling. Both the [^{35}S] labeling and the chase were performed in the presence of the drugs.

For immunoprecipitation, lysates were precleared by incubation for 1 h at 4°C with 30 ml of immobilized Protein A or protein G agarose beads coated with pre-immune rabbit or mouse sera respectively (GIBCO Lifesciences, Gaithersburg, MD). Labeled proteins were immunoprecipitated for 1 h at 4°C with protein A agarose beads chemically cross-linked to the corresponding anti-rabbit polyclonal or anti-mouse monoclonal antibodies. Certain monoclonal antibodies of the IgG_1 subclass were bound to protein G agarose beads and chemically cross-linked. In some cases, mouse monoclonal antibodies were bound to rabbit anti-mouse IgG immobilized on protein A beads. Following specific antibody binding, the immuno-beads were collected by centrifugation, washed five times with 10-20 volumes of lysis buffer lacking protease inhibitors and the labeled proteins were eluted by boiling in 50 ml of a buffer containing Tris-HCl, pH 7.4, 150 mM NaCl, 50 mM DTT, 2% SDS, glycerol (10% v/v), and bromophenol blue (0.1% w/v). The radio-labeled proteins were resolved by SDS/PAGE and visualized by conventional dry-gel autoradiography on X-Ray film followed by optical scanning or by phosphor-imaging. Radio-labeled proteins were quantitated by use of the NIH-Image program.

5. RESULTS

Acute Nef expression induces downregulation of the cell-surface expression of CD4 and its degradation in the lysosomes.

Human T cell lines expressing wt CD4 , a CD4 mutant lacking PKC susceptible serine residues, or a CD4 mutant that has lost $p56^{lck}$ association were infected either with a recombinant vaccinia virus expressing Nef (vvNef) or the control virus, vSC8. Vaccinia virus infection was monitored by FACS analysis of saponin permeabilized cells using a monoclonal antibody (TW 2.3) against the vaccinia virus E3L gene product.(93), and was routinely observed in greater than 70% of the cell population at 4 hr after infection. As illustrated in Figure 1 A, there was a significant reduction in the surface expression of wt and mutant CD4 upon expression of Nef but not with uninfected or control vaccinia virus infected cells. Similar magnitudes of CD4 downregulation was observed with vvNEF infected cells that were gated for E3L expression. Under the same conditions, Nef expression had no effect on cell-surface expression of CD2 or CD7 antigens (not shown). The loss of cell surface CD4 in the vv-Nef infected cells was accompanied by a reduction in the amount of total cellular CD4 as determined by immunoblotting (Figure 1 B). These results demonstrate that neither the dissociation of CD4 from $p56^{lck}$ nor the serines in the cytoplasmic domain of CD4 are required for the Nef effect.

Next we inquired whether the endocytosed CD4 receptor is simply sequestered in the early endosomal compartment or transported all the way to lysosomes for degradation. Receptors that are internalized and targeted to the endosomes are either recycled after delivery of the bound ligands (as with the cargo receptors) or are degraded by the acidic proteases in the lysosomes.(33, 62). The proteolysis can be prevented by treatment of cells with weak bases .(69)or protease inhibitors .(46). Following virus adsorption, the cells were treated with NH_4Cl or chloroquine, agents known to alter lysosomal pH. Figure 2 illustrates the results obtained with wt CD4. Both chloroquine and NH_4Cl treatments efficiently blocked the loss of cell surface CD4 under conditions that did not affect vaccinia virus infection. Nef induced CD4 degradation was also reversed by treating cells with proteolysis inhibitors like leupeptin (data not shown). Similar results were obtained with the non-phosphorylable triple serine mutant of CD4, the $p56^{lck}$ non-binding mutant of CD4. These results imply lysosomal degradation of CD4.

The di-leucine motif in the cytoplasmic domain of CD4 alone is not sufficient for the Nef induced endocytosis.

Recently, Aiken et al have shown that the effect on CD4 expression by Nef required a di-leucine motif in the cytoplasmic domain of CD4 .(4). To confirm these findings and to inquire whether the effect of Nef on CD4 represents a paradigm for endocytosis of receptors, we examined the effect of Nef on cell lines expressing wt CD4 or CD4 mutants that had exchanged the di-leucine motif for alanine residues or chimeric receptors containing di-leucine motifs in their cytoplasmic domain(s). As shown in Figure 3A, Nef expression failed to induce internalization of the di-leucine substitution mutant of CD4 (compare LL-413-AA with wt CD4). Depending on their location within the cytoplasmic domains of cellular proteins , di-leucine motifs have been presumed to serve as endocytotic signals in the plasma membrane and/or sorting signals in the Golgi apparatus. By analogy with CD4, the remarkably long cytoplasmic domain of EGF receptor (EGF-R) has multiple, membrane proximal di-leucine motifs .(86), one of which is preceded by a PKC sensitive threonine residue .(47). However, cell surface expression of EGF-R was remarkably insensitive to Nef coexpression (Figure 3A). Membrane distal di-leucine motifs endow the receptors with a high rate of recycling and lysosomal degradation. In their natural contexts, as in the case of lysosomal proteins, these motifs serve as trafficking signals shuttling the lysosomal proteins from the TGN to the lysosomes. We inquired whether Nef can modulate receptors containing membrane distal di-leucine motifs. For this purpose two rat basophilic leukemia cell lines .(45) expressing chimeric interleukin-2 receptors (IL-2R) were used. In the TT-γ cell line, the cytoplasmic domain of the α-chain of IL-2R was exchanged for a truncated cytoplasmic domain of the γ-chain of T-cell receptor (TCR-γ). In the DKQTLL cell line, a short stretch of -DKQTLL- was appended to the trans-membrane domain of the IL-2 α chain. Both the TT-γ and the DKQTLL chimeric receptors have high rates of turnover, resulting from lysosomal degradation .(45). The steady state surface expression of the TT-γ or the DKQTLL receptor was not modulated by Nef coexpression (Figure 3 A) in spite of the presence of a di-leucine motif. Both these chimeric receptors were not internalized after PMA treatment, much like the di-leucine substitution mutant of CD4, under conditions that induced endocytosis of EGF-R and the EGF-R/CD4 cytoplasmic domain chimera (Fig 3 B). The turnover rates of de-novo synthesized TT-γ or the -DKQTLL- receptors were not modulated by Nef under transient expression conditions in HeLa cells (data not shown). These experiments suggest that additional sequence elements besides the di-leucine motif in the cytoplasmic domain of CD4 are required for the Nef mediated downregulation.

Nef expression reduces the synthesis and the cell surface expression of nascent CD4.

Since vaccinia virus infection arrests host cellular macromolecular synthesis, the effects of Nef on CD4 described above may have reflected only the accelerated turnover of pre-synthesized receptor. To examine the effects of Nef on de-novo synthesized CD4, parallel A2.01 (CD4 negative) cultures were coinfected with a recombinant vaccinia virus encoding wt CD4 (2-5 pfu/cell) and either vvNef or control vaccinia virus. Following 30 min of virus adsorption, cells were sampled at periodic intervals and examined by flow cytometry. Both sets of vaccinia virus infected cells displayed similar levels of CD7 expression. With the vvCD4/vSC8 infected cultures, significant cell-surface expression of CD4 was detectable at 3 h post infection (p.i.) and by 6 h p.i., >60% of cells were positive for CD4 expression (Figure 4, top). In contrast, vvCD4/vvNef infected cultures lagged behind the vvCD4/vSC8 counterparts in the acquisition of CD4 surface staining, and expressed significantly less CD4 at the cell surface. At 6 h p.i. only 20% of the vvCD4/vvNef infected cells were positive for CD4 expression (Figure 4, bottom). E3L expression in both sets of co-infections matched the CD4 profiles in the vvCD4/vSC8 co-infection (not shown). At 2 h p.i., a portion of the infected cells were pulse labeled for 15 min with [^{35}S]-methionine and cysteine followed by a chase period from 0-8 hours. At the end of the pulse labeling period and at each indicated time, the labeled CD4 was immunoprecipitated and visualized by SDS/PAGE and autoradiography. As shown in Figure 4, the initial CD4 labeling in vvNef infected cells was much less than in vSC8 infected cells, suggesting that Nef affected the synthesis of CD4. Moreover, at the three hour chase, there was a significant enhancement of CD4 turnover in the cells expressing Nef.

Identification of the CD4 domains of CD4 required for the Nef mediated inhibition of CD4 biosynthesis.

The Nef induced enhancement of CD4 receptor endocytosis requires the specific presence of the CD4 cytoplasmic domain. We inquired whether the Nef mediated CD4 biosynthetic defect described above was also dictated by the cytoplasmic tail sequence. A2.01 cells were coinfected with recombinant vaccinia viruses encoding wt CD4, tCD4 or sCD4 with vvNef or control recombinant virus. At the peak of early gene expression (about 2 h p.i), the turnover rates of the respective CD4 molecules were evaluated by pulse-chase labeling experiments. Aliquots of cell lysates corresponding to equivalent levels of E3L were processed for CD4 RIPA and SDS/PAGE analysis. In the case of wt

CD4, Nef induced a two-fold decrease in the metabolic labeling of nascent CD4, followed by a rapid turnover with a $t_{1/2}$ of about 3 h. (Figure 5 A). In several experiments, the magnitude of the lower rate of CD4 biosynthesis in cells expressing Nef varied from two to six fold. This defect was specific for CD4. Nef co-expression had no effect on the biosynthesis, processing, or turnover of the unrelated CD46 protein (Figure 5B). When a recombinant vaccinia virus expressing the HIV-1 gp160 was substituted in place of CD4, Nef expression induced a mild stimulation of gp160 synthesis (Figure 5 C) that was not statistically significant. However, when gp160 was co-expressed with CD4, Nef down-regulated the CD4/gp160 complex as shown before (76), Nef expression inhibited the biosynthesis and cell surface expression of gp160 when co-expressed with CD4, by virtue of the tight association between CD4 and gp160 (data not shown).

In contrast to the results obtained with the full-length CD4, Nef induced only downregulation of tCD4 biosynthesis (Figure 5D). The levels of tCD4 detected by pulse labeling in cells infected with vvNef were reduced by 50%. However, the nascent tCD4 was properly processed (not shown) and did not exhibit any aberrant turnover (Figure 5E). FACS analysis indicated that like wt CD4, the rate of tCD4 expression at the cell surface was delayed, however, the absolute levels of tCD4 at the plasma membrane was unaffected by Nef coexpression(data not shown). Consistent with this observation was the fact that cell-surface expressed tCD4 in constitutive T cell lines was not downregulated by acute Nef expression from recombinant vaccinia virus (data not shown). Nef also reduced the biosynthetic labeling of sCD4 that lacked both the trans-membrane and cytoplasmic domains (Figure 5 E). Within the first 20 min of the chase, most of the pulse-labeled sCD4 produced in the control co-infected cells was secreted into the tissue culture supernatant, where it remains stable for several hours (unpublished data). Therefore, at each time period after the pulse labeling and during the chase, individual cell extracts were mixed with the corresponding culture fluid fractions and sCD4 was recovered by quantitative immunoprecipitation. Nef coexpression induced both a significant decrease in the early biosynthesis and downregulated the secretion of this receptor.

The Nef induced early defect of CD4 biosynthesis occurs during translation or shortly thereafter.

Next, we inquired whether the Nef induced defect in CD4 biosynthesis occurred during translation of CD4 mRNA or through a post-translational modification. The labeled proteins were resolved by SDS/PAGE and visualized by autoradiography and

quantitated by phosphor-imager scanning. As shown in Figure 6 A, Nef coexpression induced a significant reduction in CD4 biosynthesis that was discernible even after a 2 min labeling period that roughly corresponds to one or two rounds of CD4 translation. With longer labeling times there was a cumulative increase in the quantitative deficit of CD4 biosynthesis. A similar biosynthetic defect of tCD4 was also induced by Nef (Figure 6 B). In contrast, Nef expression did not inhibit the synthesis of HIV gp160 (Figure 6 C), or gp120 (not shown). An alternative explanation to the early deficit of CD4 biosynthesis in Nef coexpressing cells is the aberrant maturation of CD4 and/or protein misfolding. However, similar amounts of CD4 were recovered using polyclonal antibodies or mixtures of monoclonal antibodies. Similar differences were observed by analysis of reactivity with CD4 specific antibodies following denaturation and renaturation of CD4 molecules with SDS (not shown). Altogether, these results confirm that we were dealing with a genuine decrease in the de novo synthesis of CD4 in the Nef expressing cells. Although the labeling periods used in these experiments were too brief to allow complete protein processing and transport, we inquired whether the Nef induced early defects in CD4 production may have occurred in the ER or the Golgi apparatus. In the first scenario, agents that cause stasis of nascent glycoproteins in the ER would be expected to accentuate the Nef effect. By the same token, ER stasis would be expected to shield nascent CD4 from post Golgi degradative pathways including receptor endocytosis. To answer these questions, we treated the CD4 and Nef or vSC8 co-infected cells with the fungal metabolite, brefeldin-A (BFA), an agent that induces mixing of the Golgi and ER contents and blocks the anterograde transport of membranes and proteins from the ER to the Golgi stacks (42, 49, 54). As shown by Figure 6 A and B, BFA treatment before and during the labeling period had little on no effect on the magnitude of Nef induced defect of CD4 or tCD4 labelings. These results suggest that Nef induced defect in CD4 biosynthesis must be occurring very early in the ER.

6. DISCUSSION

Using recombinant vaccinia viruses to express Nef and/or CD4 simultaneously in T cells, we show that Nef downregulates CD4 expression by a bi-modal mechanism. Unquestionably, Nef enhanced CD4 endocytosis and this was the only demonstrable effect when Nef was expressed acutely in T cell lines that expressed CD4 constitutively. Since vaccinia infection shuts off host cell macromolecular synthesis, the effects of Nef expressed from the vaccinia vector on the endogenous CD4 synthesis could not be measured. However, when Nef and CD4 were co-expressed from recombinant vaccinia viruses in cells lacking endogenous CD4, we demonstrated a Nef induced defect in the biosynthesis of CD4 in addition to the accelerated endocytosis of the CD4 receptor.

In confirmation of earlier reports (4, 74), we have demonstrated that the enhanced endocytosis of the CD4 receptor induced by Nef requires the presence of a di-leucine motif in the cytoplasmic tail of CD4. Our results also show that the -L-L- motif is not sufficient to confer sensitivity to Nef induced endocytosis. Indeed, Nef did not modulate endocytosis of EGF-R, TT-γ or DQTLL receptors all of which have dileucine motifs. It is possible that the dileucine motif is required for targeting the protein to the lysosomal compartment, but other sequences such as those constituting the amphipathic helix in the CD4 cytoplasmic tail (79) are also required for internalization of the receptor into the clathrin coated vesicles. This is consistent with the recent demonstration that a -EMKL- sequence, upstream of the dileucine motif of CD4 is required for the Nef mediated endocytosis ((74), S.Gratton, unpublished data).

The cytoplasmic domains of many receptors contain sequence determinants that specify the subcellular organelle targeting or intracellular transport. Two sequence motifs, namely, the invariant tyrosine residue and membrane proximal or distal di-leucine motifs have been implicated in these targeting pathways. When the di-leucine motifs occur near the C-terminus as in the mannose 6-phosphate/insulin-like growth factor-II receptor (16, 38), the cation dependent mannose-6-phosphate receptor (39), or the lysosomal integral membrane protein (LIMP II) (87, 88) , they are presumed to interact with the Golgi adaptor proteins independently of serine/threonine phosphorylation (38, 39, 87, 88) and routed to the clathrin coated vesicles which bud off from the Golgi network and are sorted into the pre-lysosomal compartments. Membrane proximal di-leucine motifs occur in the cytoplasmic domains of numerous receptors including CD4 (79, 80), CD3 γ and δ chains (19, 45), the interferon γ receptor (26), the EGF receptor (47, 86) and other trans-membrane tyrosine kinase receptors. In many of these cases including CD4, the di-leucine

motif is presumed to be inaccessible to the endocytotic apparatus; phosphorylation of adjacent serine or threonine residues following PMA activation or conformational change following ligand binding and receptor oligomerization are thought to initiate endocytosis by exposing the dileucine motif to interaction with adaptins assembled at the clathrin coated pits.(19, 45).

The fact that the triple serine mutant remains sensitive to Nef while it is not downregulated by PMA clearly indicates that these endocytotic pathways are different. We cannot formally exclude the possibility that Nef may facilitate CD4 internalization by inducing phosphorylation of other Ser or Thr residues in the cytoplasmic tail of CD4. It is of interest to note that Nef associates with a putative 80 KDa serine/threonine kinase.(75). Although amino-acid substitution of all the three serines in CD4 (our PMA non-responsive 3 S mutant) and mutation of the threonine at 418 preserved the Nef response.(32, 74), the phenotype of mutating all the serines and the threonines in the cytoplasmic tail of CD4 is not known. It is equally interesting that the CD4 mutant which does not associate with $p56^{lck}$, is still downregulated by Nef suggesting that this *src* related tyrosine kinase is not directly involved in the downregulation of cell-surface CD4. It is likely, however, that CD4 degradation leads to an increase in non-CD4 associated $p56^{lck}$ favoring T-cell activation and HIV replication (S. Gratton, unpublished data).

Our data also demonstrates a second aspect of CD4 downregulation. In both T cells and non-lymphoid cells, Nef induced early (co-translational or immediate post-translational) defect in CD4 biosynthesis. The early defect was specific for CD4; unrelated cellular or viral surface glycoproteins like CD46, HIV-1 *env* gp160 and influenza virus HA (not shown) proteins were unaffected by Nef expression. The early defect requires a CD4 sequence other than the TM and the Cyt domains, since both t-CD4 (w/o Cyt domain) and the soluble s-CD4 (w/o Cyt & TM domains) were downregulated in a similar manner. More direct proof for an early defect was obtained in short term labeling experiments. There was a consistent quantitative deficit of nascent CD4 in Nef expressing cells that was detectable during a 2 min pulse labeling. Similar results were obtained using either poly-clonal or two different monoclonal antibodies against CD4, suggesting that this observation is not due to reduced immuno-reactivity of nascent CD4 resulting from aberrant or delayed protein folding. Also, the apparent defect should disappear during the "chase" when sufficient time has elapsed to allow for correct protein folding. But, the early CD4 deficit persisted during the transit into the ER, and the Golgi. Nef did not significantly decrease the half-life of nascent CD4 that had already entered the ER (except at later times). Furthermore, Nef did not interfere with the rate of CD4 processing when

analyzed by acquisition of endoglycosidase H resistance (not shown). The defect in CD4 labeling persisted during BFA treatment which is known to block the anterograde-grade transport of proteins and membranes from the ER to the Golgi apparatus.(42, 49, 54) and therefore prevent the endocytosis of nascent proteins.

The CD4 biosynthetic defect may be mediated by Nef effects on polypeptide translation.(70), or on intracellular vesicular traffic of nascent proteins. While we have observed a Nef induced CD4 translation defect *in vitro* (Hiller, S., unpublished data), we have been unable to correlate this defect to specific sub-domains of CD4. However, there is some precedent to suspect that Nef may affect vesicular traffic out of the ER. Recently, a direct physical interaction of Nef with the β-COP coatamer subunit component of non-clathrin-coated vesicles has been demonstrated.(9). β-COP is an essential component of the non-selective vesicular transport system from the ER to the Golgi, between the Golgi cisternae, and from the TGN to the plasma membrane.(20, 22, 44, 61, 66, 67). In transient transfections, we observed colocalization of β-COP and Nef, and depending on the levels of Nef expression, there was fragmentation and dissolution of coatamer coated vesicles (Venkatesan, S. unpublished data). Although abrupt perturbation of vesicular traffic by interference with β-COP assembly may be expected to have a global effect on the transport of macromolecules out of the ER, and argue against the specificity of the Nef effect on CD4, Nef may simply delay the formation of these vesicular complexes or the recruitment of selected nascent proteins into these vesicles. As a consequence, acquisition of nascent CD4 by the coatamer vesicles may be delayed sufficiently long for the recruitment of CD4 by cellular chaperones and/or proteases. Alternatively, Nef may directly or indirectly interfere with the transport of CD4 into pre-Golgi vesicles. This may result from a simple ternary interaction between Nef, β-COP, and CD4, although we cannot eliminate other potential interactions. Nef lacks a signal sequence or a definable trans-membrane domain; but co-translational myristoylation of Nef may insert it into the ER membrane and allow it to interact with CD4. It is significant to note that the Nef effect on CD4 biosynthesis both in the vaccinia expression system and during transient transfections (Hiller et al, unpublished data) was dose-dependent on Nef expression. This may simply reflect thresho!' requirements of Nef for interference with the vesicular traffic. β-COP has significant homology to the β-adaptin component of the clathrin-coated vesicles.(22),and it is conceivable that Nef may potentially interact with the β-adaptins as well. Although no candidate molecule other than CD4 has been demonstrated to be affected by Nef by virtue of interaction with the key components of coated vesicles, Nef may be expected to modulate both the antero-grade transport and endocytosis of other unidentified proteins as

well. However, since endocytosis is mediated by specific signal sequence within the particular receptor molecules, Nef may only modulate a limited subset of plasma membrane proteins.

Finally, a few comments about the relevance of CD4 downregulation by Nef in HIV life cycle should be made. Nef is an early gene product (2, 27) and SIV Nef expressing T cell lines have been shown to be resistant to super-infection by SIV (11). Similarly, Nef induced loss of CD4 receptors early during acute infection will be expected to moderate the infectious process and/or induce chronic infection. However, even within the first round of virus replication, expression of the HIV Vpu and env gp160 proteins can induce further loss of the CD4 receptor with similar negative consequence for super-infection. Further, the requirement of a competent Nef gene for the maintenance of virus load in the SIVmac/rhesus monkey system (40) suggests that Nef may potentiate a positive effect on virus replication in infected animals by inducing T cell activation. Although by inducing CD4 endocytosis, Nef may release the CD4 associated $p56^{lck}$ from its normal physiological functional constraints, the functional consequences of this process *in vivo* are unclear. There have been conflicting reports as to whether the $p56^{lck}$ dissociation following virus entry of T cells results in a negative or positive effect on cellular transcriptional factors (10, 84). Further complicating this scenario is the recent report showing that depending on its subcellular localization, Nef activates or inhibits signalling events from the T cell receptor (8). However, this report did not correlate these opposite phenotypes associated with Nef expression to CD4 receptor downregulation. Thus it is reasonable to conclude that CD4 downregulation may be just be one of the many effects of Nef on cellular regulation and caution should be exercised before extrapolating the effect on CD4 to diverse processes such as T cell activation, cell signalling and transcriptional regulation.

FIGURE LEGENDS

Figure 1 : Nef induces down-regulation of cell surface expression of CD4 and mutants of CD4. **A)** FACS analysis of the effects of Nef and PMA on the cell-surface expression of CD4 in human T cell lines expressing wild-type CD4 (**wt**), a CD4 mutant that substituted the Cys residues for Ala at positions 420 and 422 and lacked $p56^{lck}$ binding (**CC 420 AA**), and a mutant that substituted all the Ser residues in the cytoplasmic domain of CD4 (**CD4 3S**). In each case, approximateiy 10^6 cells were left untreated (**UI**), or infected with the recombinant vaccinia viruses, **vSC8** or **vvNef** at a m.o.i of 5 pfu/cell as described under the **Experimental Procedures**. At 4 h after infection, separate 10^5 cell aliquots were stained for the surface expression of CD7 and CD4 using PE or FITC conjugated monoclonal antibodies respectively. Results obtained with the FITC conjugated CD4 antibodies are shown with the respective mean fluorescent values (**MFV**). CD4 expression following PMA treatment was analyzed 1 h after treatment of the cells with 5×10^{-8} M PMA. FACS profiles of untreated and PMA treated cells are denoted by hashed and continuous plots respectively. **B)** Immunoblot detection of CD4 and Nef in the respective infections above.

Figure 2 : Nef induced down-regulation of cell-surface CD4 is reversed by NH_4Cl or chloroquine (CHQ) treatment. Human T cell lines expressing wt CD4 were analyzed for CD4 expression by FACS as described in Figure 1. Where indicated, the cells were treated with NH_4CL (75 mM) or CHQ (100 mM) starting at 30 min after virus adsorption. In each case, the progress of vaccinia infection was monitored by FACS using a vaccinia specific monoclonal antibody, E3L. FACS profiles of CD7 were used as negative controls (not shown)

Figure 3: A) A di-leucine motif in the cytoplasmic domain of CD4 is required, but is not sufficient to confer sensitivity to downregulation by Nef. Human T cell lines expressing native CD4 (**wt**), or a mutant CD4 that has the two leucines at positions 413-414 substituted for alanines (**LL-413-AA**), and a CD4 negative T cell line expressing wild-type human EGF receptor (**EGF-R1**) were for utilized to examine the Nef effect on the surface expression of the respective receptor(s). Rat basophilic leukemia cell lines expressing a chimera that fused the C-terminus of the IL2 receptor alpha chain to a truncated version of the cytoplasmic domain of human T cell receptor gamma chain (**IL2-TTg**) or to a synthetic -DKQTLL motif (**IL2R-DKQTLL**) were used to examine the role of the dileucine motif in a non-natural context. Individual cell lines were infected with the Nef recombinant (**vv-Nef**) or a control vaccinia virus (**vSC8**) at an m.o.i of 5

pfu/cell, or left uninfected (UI). The cells were processed for FACS analysis at 4 h after infection. Direct staining with PE conjugated anti-Leu3A CD4 monoclonal antibody was used to detect CD4; chimeric IL2 receptor was detected by use of a mouse monoclonal antibody, 7G7 (Letourneur et al, 1991), and the EGF-R1 ecto-domain by the use of a commercial anti-EGF receptor monoclonal antibody (Upstate Biotechnologies, Lake Placid, NY), followed by PE-conjugated rabbit anti-mouse IgG staining. B) PMA treatment downregulated wt CD4, EGF-R and a EGF-R/CD4 chimera that fused the CD4 TM and CYT domains to the EGF-R ectodomain, but not the LL-413-AA CD4 mutant or the chimeric IL-2 receptors containing a cytoplasmic di-leucine motif. The indicated cell lines were either treated with 10^{-8} M PMA or left untreated for 1 hr, and then processed for FACS analysis using the respective monoclonal antibodies as shown.

Figure 4 : Time-course of CD4 biosynthesis in A2.01 cells coinfected with a recombinant vaccinia virus encoding CD4 and either vvNef or vSC8 or WR. A2.01 T cell lines were coinfected with a recombinant vaccinia virus encoding CD4 and vSC8 control recombinant (top) or vvNef (bottom) at a total m.o.i of 10 pfu per cell. Aliquots of cells were analyzed at the indicated times for cell surface expression of CD4 . At 2 h after virus adsorption, 10^7 cells were recovered from each co-infection, starved for 10 min in methionine free medium and pulse-labeled for 15 min with [^{35}S]-Trans-label (ICN Biochem) followed by a metabolic chase. Samples were taken at the end of pulse and the indicated chase periods. After adjusting for equivalent vaccinia infection, the aliquots were processed for CD4 immunoprecipitation. The pulse chase results for each infection are shown underneath their respective FACS profiles.

Figure 5 : Pulse chase analysis of CD4 turnover in the presence or absence of Nef. A2.01 cells were infected with equal m.o.i. of a recombinant vaccinia virus expressing wt CD4, vCD4 together with either the Nef recombinant (vvNef) or control virus (vSC8) (**A**). The recombinant vaccinia virus expressing the human CD46 was included as an additional control in some of the CD4 or tCD4 and Nef, and CD4 or tCD4 and vSC8 infections (panel **B**). In separate experiments, HIV-1 gp160 expression from a vaccinia recombinant was also evaluated in the presence or absence of Nef (panel **C**). In **D**, and **E**, vaccinia recombinants vaccinia viruses expressing tailless CD4 (tCD4) or the secretory form of CD4 (sCD4) were substituted for wt CD4 virus respectively. Cells were labeled and the cell extracts were processed for vaccinia expressed recombinant proteins as described in **Experimental Procedures.** In each case, Nef and vSC8 infected cell lysates were normalized to E3L expression . In the case of sCD4 infections (panel **C**), cell extracts for individual time points were mixed with the corresponding culture

fluid fractions and the combined intracellular and secreted fractions of sCD4 were recovered by immuno-precipitation. CD46 expression was detected using a commercial monoclonal antibody (panel **D**). A representative of at least three (tCD4 and s-CD4)and as many as eight (wt CD4) independent experiments is shown for each set.

Figure 6 : Short term labeling of CD4 (panel **A**), tCD4 (panel **B**), HIV-1 gp160 (panel **C**), expressed from the respective vaccinia recombinants in the presence or the absence of Nef co-expression. A2.01 cells were coinfected with the respective vaccinia recombinants and Nef or the control virus vSC8. At 4 h after infection, cells were labeled for the indicated times and processed as described in **Experimental Procedures**, and a representative of three experiments is shown. In the case of vCD4 and tCD4 experiments, parallel sets of vSC8 and vvNEF co-infections were treated for 1 h with BFA (2 mg/ml) before and during the labeling periods (+ **BFA**).

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7. REFERENCES

.1. **Acres, R. B., P. J. Conlon, D. Y. Mochizuki, and B. Gallis.** 1986. Rapid phosphorylation and modulation of the T4 antigen on cloned helper T cells induced by phorbol myristate acetate or antigen. J Biol Chem. **261**:16210-4.

2. **Ahmad, N., R. Maitra, and S. Venkatesan.** 1989. Rev-induced modulation of Nef protein underlies temporal regulation of human immunodeficiency virus replication. Proc Natl Acad Sci U S A. **86**:6111-5.

3. **Ahmad, N., and S. Venkatesan.** 1988. Nef Protein of HIV-1 is a Transcriptional Repressor of HIV-1 LTR. Science. **241**:1481-1485.

4. **Aiken, C., J. Konner, N. R. Landau, M. E. Lenburg, and D. Trono.** 1994. Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. Cell. **76**:853-64.

5. **Allan, J. S., J. E. Coligan, T. H. Lee, M. F. McLane, P. J. Kanki, J. E. Groopman, and M. Essex.** 1985. A new HTLV-III/LAV encoded antigen detected by antibodies from AIDS patients. Science. **230**:810-3.

6. **Anderson, S., D. C. Shugars, R. Swanstrom, and J. V. Garcia.** 1993. Nef from primary isolates of human immunodeficiency virus type 1 suppresses surface CD4 expression in human and mouse T cells. J Virol. **67**:4923-31.

7. **Barber, E. K., J. D. Dasgupta, S. F. Schlossman, J. M. Trevillyan, and C. E. Rudd.** 1989. The CD4 and CD8 antigens are coupled to a protein-tyrosine kinase (p56lck) that phosphorylates the CD3 complex. Proc Natl Acad Sci U S A. **86**:3277-81.

8. **Baur, A. S., E. T. Sawai, P. Dazin, W. J. Fantl, C. Cheng-Mayer, and B. M. Peterlin.** 1994. HIV-1 Nef leads to inhibition or activation of T cells depending on its intracellular localization. Immunity. **1**:373-84.

9. **Benichou, S., M. Bomsel, M. Bodeus, H. Durand, M. Doute, F. Letourneur, J. Camonis, and R. Benarous.** 1994. Physical interaction of the HIV-1 Nef protein with beta-COP, a component of non-clathrin-coated vesicles essential for membrane traffic. J Biol Chem. **269**:30073-6.

10. **Benkirane, M., K. T. Jeang, and C. Devaux.** 1994. The cytoplasmic domain of CD4 plays a critical role during the early stages of HIV infection in T-cells. Embo J. **13**:5559-69.

11. **Benson, R. E., A. Sanfridson, J. S. Ottinger, C. Doyle, and B. R. Cullen.** 1993. Downregulation of cell-surface CD4 expression by simian immunodeficiency virus Nef prevents viral super infection. J Exp Med. **177**:1561-6.

12. **Brady, H. J., D. J. Pennington, C. G. Miles, and E. A. Dzierzak.** 1993. CD4 cell surface downregulation in HIV-1 Nef transgenic mice is a consequence of intracellular sequestration. Embo J. **12**:4923-32.

13. **Broder, C. C., and E. A. Berger.** 1993. CD4 molecules with a diversity of mutations encompassing the CDR3 region efficiently support human immunodeficiency virus type 1 envelope glycoprotein-mediated cell fusion. J Virol. **67**:913-26.

14. **Broder, C. C., D. S. Dimitrov, R. Blumenthal, and E. A. Berger.** 1993. The block to HIV-1 envelope glycoprotein-mediated membrane fusion in animal cells expressing human CD4 can be overcome by a human cell component(s). Virology. **193**:483-91.

15. **Chakrabarti, S., K. Brechling, and B. Moss.** 1985. Vaccinia virus expression vector: coexpression of beta-galactosidase provides visual screening of recombinant virus plaques. Mol Cell Biol. **5**:3403-9.

16. **Chen, H. J., J. Remmler, J. C. Delaney, D. J. Messner, and P. Lobel.** 1993. Mutational analysis of the cation-independent mannose 6-phosphate/insulin-like growth factor II receptor. A consensus casein kinase II site followed by 2 leucines near the carboxyl terminus is important for intracellular targeting of lysosomal enzymes [published erratum appears in J Biol Chem 1994 Jun 10;269(23):16518]. J Biol Chem. **268**:22338-46.

17. **Chowers, M. Y., C. A. Spina, T. J. Kwoh, N. J. Fitch, D. D. Richman, and J. C. Guatelli.** 1994. Optimal infectivity in vitro of human immunodeficiency virus type 1 requires an intact nef gene. J Virol. **68**:2906-14.

18. **Delassus, S., R. Cheynier, and H. S. Wain.** 1991. Evolution of human immunodeficiency virus type 1 nef and long terminal repeat sequences over 4 years in vivo and in vitro. J Virol. **65**:225-31.

19. **Dietrich, J., X. Hou, A. M. Wegener, and C. Geisler.** 1994. CD3 gamma contains a phosphoserine-dependent di-leucine motif involved in down-regulation of the T cell receptor. Embo J. **13**:2156-66.

20. **Donaldson, J. G., J. Lippincott-Schwartz, G. S. Bloom, T. E. Kreis, and R. D. Klausner.** 1990. Dissociation of a 110-kD peripheral membrane protein from the Golgi apparatus is an early event in brefeldin A action. J Cell Biol. **111**:2295-306.

21. **Doyle, C., and J. L. Strominger.** 1987. Interaction between CD4 and class II MHC molecules mediates cell adhesion. Nature. **330**:256-9.

22. **Duden, R., G. Griffiths, R. Frank, P. Argos, and T. E. Kreis.** 1991. Beta-COP, a 110 kd protein associated with non-clathrin-coated vesicles and the Golgi complex, shows homology to beta-adaptin. Cell. **64**:649-65.

23. **Earl, P. L., R. W. Doms, and B. Moss.** 1990. Oligomeric structure of the human immunodeficiency virus type 1 envelope glycoprotein. Proc Natl Acad Sci U S A. **87**:648-52.

24. **Earl, P. L., A. W. H:ugin, and B. Moss.** 1990. Removal of cryptic poxvirus transcription termination signals from the human immunodeficiency virus type 1 envelope gene enhances expression and immunogenicity of a recombinant vaccinia virus. J Virol. **64**:2448-51.

25. **Earl, P. L., M. Robert-Guroff, T. J. Matthews, K. Krohn, W. T. London, and B. Moss.** 1989. Isolate- and group-specific immune responses to the envelope protein of human immunodeficiency virus induced by a live recombinant vaccinia virus in macaques. Aids Res Hum Retroviruses. **5**:23-32.

26. **Farrar, M. A., J. Fernandez-Luna, and R. D. Schreiber.** 1991. Identification of two regions within the cytoplasmic domain of the human interferon-gamma receptor required for function. J Biol Chem. **266**:19626-35.

27. **Felber, B. K., C. M. Drysdale, and G. N. Pavlakis.** 1990. Feedback regulation of human immunodeficiency virus type 1 expression by the Rev protein. J Virol. **64**:3734-41.

28. **Franchini, G., M. Robert-Guroff, J. Ghrayeb, N. T. Chang, and F. Wong-Staal.** 1986. Cytoplasmic localization of the HTLV-III 3' orf protein in cultured T cells. Virology. **155**:593-9.

29. **Franchini, G., M. Robert-Guroff, F. Wong-Staal, J. Ghrayeb, I. Kato, T. W. Chang, and N. T. Chang.** 1986. Expression of the protein encoded by the 3' open reading frame of human T-cell lymphotropic virus type III in bacteria: demonstration of its immunoreactivity with human sera. Proc Natl Acad Sci U S A. **83**:5282-5.

30. **Garcia, J. V., J. Alfano, and A. D. Miller.** 1993. The negative effect of human immunodeficiency virus type 1 Nef on cell surface CD4 expression is not species specific and requires the cytoplasmic domain of CD4. J Virol. **67**:1511-6.

31. **Garcia, J. V., and A. D. Miller.** 1992. Downregulation of cell surface CD4 by nef. Res Virol. **143**:52-5.

32. **Garcia, J. V., and A. D. Miller.** 1991. Serine phosphorylation-independent downregulation of cell-surface CD4 by nef. Nature. **350**:508-11.

33. **Goldstein, J. L., M. S. Brown, R. G. Anderson, D. W. Russell, and W. J. Schneider.** 1985. Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. Annu Rev Cell Biol. **1**:1-39.

34. **Greenway, A. L., D. A. McPhee, E. Grgacic, D. Hewish, A. Lucantoni, I. Macreadie, and A. Azad.** 1994. Nef 27, but not the Nef 25 isoform of human immunodeficiency virus-type 1 pNL4.3 down-regulates surface CD4 and IL-2R expression in peripheral blood mononuclear cells and transformed T cells. Virology. **198**:245-56.

35. **Guy, B., M. P. Kieny, Y. Riviere, C. Le Peuch, K. Dott, M. Girard, L. Montagnier, and J. P. Lecocq.** 1987. HIV F/3' orf encodes a phosphorylated GTP-binding protein resembling an oncogene product. Nature. **330**:266-9.

36. **Hammes, S. R., E. P. Dixon, M. H. Malim, B. R. Cullen, and W. C. Greene.** 1989. Nef protein of human immunodeficiency virus type 1: evidence against its role as a transcriptional inhibitor. Proc Natl Acad Sci U S A. **86**:9549-53.

37. **Hurley, T. R., K. Luo, and B. M. Sefton.** 1989. Activators of protein kinase C induce dissociation of CD4, but not CD8, from p56lck. Science. **245**:407-9.

38. **Johnson, K. F., and S. Kornfeld.** 1992. The cytoplasmic tail of the mannose 6-phosphate/insulin-like growth factor-II receptor has two signals for lysosomal enzyme sorting in the Golgi. J Cell Biol. **119**:249-57.

39. **Johnson, K. F., and S. Kornfeld.** 1992. A His-Leu-Leu sequence near the carboxyl terminus of the cytoplasmic domain of the cation-dependent mannose 6-phosphate receptor is necessary for the lysosomal enzyme sorting function. J Biol Chem. **267**:17110-5.

40. **Kestler, H. d., D. J. Ringler, K. Mori, D. L. Panicali, P. K. Sehgal, M. D. Daniel, and R. C. Desrosiers.** 1991. Importance of the nef gene for maintenance of high virus loads and for development of AIDS. Cell. **65**:651-62.

41. **Kim, S., K. Ikeuchi, R. Byrn, J. Groopman, and D. Baltimore.** 1989. Lack of a negative influence on viral growth by the nef gene of human immunodeficiency virus type 1. Proc Natl Acad Sci U S A. **86**:9544-8.

42. **Klausner, R. D., J. G. Donaldson, and J. Lippincott-Schwartz.** 1992. Brefeldin A: insights into the control of membrane traffic and organelle structure. J Cell Biol. **116**:1071-80.

43. **Koenig, S., T. R. Fuerst, L. V. Wood, R. M. Woods, J. A. Suzich, G. M. Jones, V. F. de la Cruz, R. Davey, Jr., S. Venkatesan, B. Moss, and a. et.** 1990. Mapping the fine specificity of a cytolytic T cell response to HIV-1 nef protein. J Immunol. **145**:127-35.

44. **Kreis, T. E., and R. Pepperkok.** 1994. Coat proteins in intracellular membrane transport. Curr Opin Cell Biol. **6**:533-7.

45. **Letourneur, F., and R. D. Klausner.** 1992. A novel di-leucine motif and a tyrosine-based motif independently mediate lysosomal targeting and endocytosis of CD3 chains. Cell. **69**:1143-57.

46. **Libby, P., and A. L. Goldberg.** 1978. Leupeptin, a protease inhibitor, decreases protein degradation in normal and diseased muscles. Science. **199**:534-536.

47. **Lin, C. R., W. S. Chen, C. S. Lazar, C. D. Carpenter, G. N. Gill, R. M. Evans, and M. G. Rosenfeld.** 1986. Protein kinase C phosphorylation at Thr 654 of the unoccupied EGF receptor and EGF binding regulate functional receptor loss by independent mechanisms. Cell. **44**:839-48.

48. **Lindemann, D., R. Wilhelm, P. Renard, A. Althage, R. Zinkernagel, and J. Mous.** 1994. Severe immunodeficiency associated with a human immunodeficiency virus 1 NEF/3'-long terminal repeat transgene. J Exp Med. **179**:797-807.

49. **Lippincott-Schwartz, J., L. Yuan, C. Tipper, M. Amherdt, L. Orci, and R. D. Klausner.** 1991. Brefeldin A's effects on endosomes, lysosomes, and the TGN suggest a general mechanism for regulating organelle structure and membrane traffic. Cell. **67**:601-16.

50. **Luciw, P. A., C. Cheng-Mayer, and J. A. Levy.** 1987. Mutational analysis of the human immunodeficiency virus: the orf-B region down-regulates virus replication. Proc Natl Acad Sci U S A. **84**:1434-8.

51. **Luria, S., I. Chambers, and P. Berg.** 1991. Expression of the type 1 human immunodeficiency virus Nef protein in T cells prevents antigen receptor-mediated induction of interleukin 2 mRNA. Proc Natl Acad Sci U S A. **88**:5326-30.

52. **Miceli, M. C., and J. R. Parnes.** 1993. Role of CD4 and CD8 in T cell activation and differentiation. Adv Immunol. **53**:59-122.

53. **Miller, M. D., M. T. Warmerdam, I. Gaston, W. C. Greene, and M. B. Feinberg.** 1994. The human immunodeficiency virus-1 nef gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages. J Exp Med. **179**:101-13.

54. **Misumi, Y., Y. Misumi, K. Miki, A. Takatsuki, G. Tamura, and Y. Ikehara.** 1986. Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. J Biol Chem. **261**:11398-403.

55. **Muesing, M. A., D. H. Smith, C. D. Cabradilla, C. V. Benton, L. A. Lasky, and D. J. Capon.** 1985. Nucleic acid structure and expression of the human AIDS/lymphadenopathy retrovirus. Nature. **313**:450-8.

56. **Niederman, T. M., J. V. Garcia, W. R. Hastings, S. Luria, and L. Ratner.** 1992. Human immunodeficiency virus type 1 Nef protein inhibits NF-kappa B induction in human T cells. J Virol. **66**:6213-9.

57. **Niederman, T. M., W. R. Hastings, S. Luria, J. C. Bandres, and L. Ratner.** 1993. HIV-1 Nef protein inhibits the recruitment of AP-1 DNA-binding activity in human T-cells. Virology. **194**:338-44.

58. **Niederman, T. M., W. Hu, and L. Ratner.** 1991. Simian immunodeficiency virus negative factor suppresses the level of viral mRNA in COS cells. J Virol. **65**:3538-46.

59. **Niederman, T. M., B. J. Thielan, and L. Ratner.** 1989. Human immunodeficiency virus type 1 negative factor is a transcriptional silencer. Proc Natl Acad Sci U S A. **86**:1128-32.

60. **Nussbaum, O., C. C. Broder, B. Moss, L. B.-L. Stern, S. Rozenblatt, and E. A. Berger.** 1995. Functional and Structural Interactions between Measles Virus Hemagglutinin and CD4. J. Virol. **69**:3341-3349.

61. **Oprins, A., R. Duden, T. E. Kreis, H. J. Geuze, and J. W. Slot.** 1993. Beta-COP localizes mainly to the cis-Golgi side in exocrine pancreas. J Cell Biol. **121**:49-59.

62. **Pearse, B. M. F., and M. S. Robinson.** 1990. Clathrin, Adaptors and Sorting. Annu Rev Cell Biol. **6**:151-171.

63. **Pelchen-Matthews, A., J. E. Armes, G. Griffiths, and M. Marsh.** 1991. Differential endocytosis of CD4 in lymphocytic and nonlymphocytic cells. J Exp Med. **173**:575-87.

64. **Pelchen-Matthews, A., I. Boulet, D. R. Littman, R. Fagard, and M. Marsh.** 1992. The protein tyrosine kinase p56lck inhibits CD4 endocytosis by preventing entry of CD4 into coated pits. J Cell Biol. **117**:279-90.

65. **Pelchen-Matthews, A., I. J. Parsons, and M. Marsh.** 1993. Phorbol ester-induced downregulation of CD4 is a multistep process involving dissociation from p56lck, increased association with clathrin-coated pits, and altered endosomal sorting. J Exp Med. **178**:1209-22.

66. **Pepperkok, R., J. Scheel, H. Horstmann, H. P. Hauri, G. Griffiths, and T. E. Kreis.** 1993. Beta-COP is essential for biosynthetic membrane transport from the endoplasmic reticulum to the Golgi complex in vivo. Cell. **74**:71-82.

67. **Peter, F., H. Plutner, H. Zhu, T. E. Kreis, and W. E. Balch.** 1993. Beta-COP is essential for transport of protein from the endoplasmic reticulum to the Golgi in vitro. J Cell Biol. **122**:1155-67.

68. **Peterson, A., and B. Seed.** 1988. Genetic analysis of monoclonal antibody and HIV binding sites on the human lymphocyte antigen CD4. Cell. **54**:65-72.

69. **Poole, B., and S. Ohkuma.** 1981. Effect of weak bases on the intralysosomal pH in mouse peritoneal macrophages. J Cell Biol. **90**:665-9.

70. **Poulin, L., M. Fauchon, A. Darveau, and J. A. Levy.** 1994. Inhibition of protein synthesis by the human immunodeficiency virus type 1 nef gene product. J Gen Virol. **75**:2977-84.

71. **Rhee, S. S., and J. W. Marsh.** 1994. HIV-1 Nef activity in murine T cells. CD4 modulation and positive enhancement. J Immunol. **152**:5128-34.

72. **Robey, E., and R. Axel.** 1990. CD4: collaborator in immune recognition and HIV infection. Cell. **60**:697-700.

73. **Rudd, C. E., J. M. Trevillyan, J. D. Dasgupta, L. L. Wong, and S. F. Schlossman.** 1988. The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (pp58) from human T lymphocytes. Proc Natl Acad Sci U S A. **85**:5190-4.

74. **Salghetti, S., R. Mariani, and J. Skowronski.** 1995. Human immunodeficiency virus type 1 Nef and p56lck protein-tyrosine kinase interact with a common element in CD4 cytoplasmic tail. Proc Natl Acad Sci U S A. **92**:349-53.

75. **Sawai, E. T., A. Baur, H. Struble, B. M. Peterlin, J. A. Levy, and C. Cheng-Mayer.** 1994. Human immunodeficiency virus type 1 Nef associates with a cellular serine kinase in T lymphocytes. Proc Natl Acad Sci U S A. **91**:1539-43.

76. **Schwartz, O., Y. Riviere, J. M. Heard, and O. Danos.** 1993. Reduced cell surface expression of processed human immunodeficiency virus type 1 envelope glycoprotein in the presence of Nef. J Virol. **67**:3274-80.

77. **Schwartz, S., B. K. Felber, D. M. Benko, E. M. Feny:o, and G. N. Pavlakis.** 1990. Cloning and functional analysis of multiply spliced mRNA species of human immunodeficiency virus type 1. J Virol. **64**:2519-29.

78. **Shaw, A. S., K. E. Amrein, C. Hammond, D. F. Stern, B. M. Sefton, and J. K. Rose.** 1989. The lck tyrosine protein kinase interacts with the cytoplasmic tail of the CD4 glycoprotein through its unique amino-terminal domain. Cell. **59**:627-36.

79. **Shin, J., C. Doyle, Z. Yang, D. Kappes, and J. L. Strominger.** 1990. Structural features of the cytoplasmic region of CD4 required for internalization. Embo J. **9**:425-34.

80. **Shin, J., R. Dunbrack, Jr., S. Lee, and J. L. Strominger.** 1991. Phosphorylation-dependent down-modulation of CD4 requires a specific structure within the cytoplasmic domain of CD4. J Biol Chem. **266**:10658-65.

81. **Skowronski, J., D. Parks, and R. Mariani.** 1993. Altered T cell activation and development in transgenic mice expressing the HIV-1 nef gene. Embo J. **12**:703-13.

82. **Spina, C. A., T. J. Kwoh, M. Y. Chowers, J. C. Guatelli, and D. D. Richman.** 1994. The importance of nef in the induction of human immunodeficiency

virus type 1 replication from primary quiescent CD4 lymphocytes. J Exp Med. **179**:115-23.

83. **Terwilliger, E., J. G. Sodroski, C. A. Rosen, and W. A. Haseltine.** 1986. Effects of mutations within the 3' orf open reading frame region of human T-cell lymphotropic virus type III (HTLV-III/LAV) on replication and cytopathogenicity. J Virol. **60**:754-60.

84. **Tremblay, M., S. Meloche, S. Gratton, M. A. Wainberg, and R. P. Sekaly.** 1994. Association of p56lck with the cytoplasmic domain of CD4 modulates HIV-1 expression. Embo J. **13**:774-83.

85. **Turner, J. M., M. H. Brodsky, B. A. Irving, S. D. Levin, R. M. Perlmutter, and D. R. Littman.** 1990. Interaction of the unique N-terminal region of tyrosine kinase p56lck with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. Cell. **60**:755-65.

86. **Ullrich, A., L. Coussens, J. S. Hayflick, T. J. Dull, A. Gray, A. W. Tam, J. Lee, Y. Yarden, T. A. Libermann, J. Schlessinger, and et al.** 1984. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature. **309**:418-25.

87. **Vega, M. A., F. Rodriguez, B. Segui, C. Cales, J. Alcalde, and I. V. Sandoval.** 1991. Targeting of lysosomal integral membrane protein LIMP II. The tyrosine-lacking carboxyl cytoplasmic tail of LIMP II is sufficient for direct targeting to lysosomes. J Biol Chem. **266**:16269-72.

88. **Vega, M. A., B. Segui-Real, J. A. Garcia, C. Cales, F. Rodriguez, J. Vanderkerckhove, and I. V. Sandoval.** 1991. Cloning, sequencing, and expression of a cDNA encoding rat LIMP II, a novel 74-kDa lysosomal membrane protein related to the surface adhesion protein CD36. J Biol Chem. **266**:16818-24.

89. **Veillette, A., M. A. Bookman, E. M. Horak, and J. B. Bolen.** 1988. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56lck. Cell. **55**:301-8.

90. **Veillette, A., M. A. Bookman, E. M. Horak, L. E. Samelson, and J. B. Bolen.** 1989. Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase p56lck. Nature. **338**:257-9.

91. **Weiss, A., B. A. Irving, L. K. Tan, and G. A. Koretzky.** 1991. Signal transduction by the T cell antigen receptor. Semin Immunol. **3**:313-24.

92. **Weyand, C. M., J. Goronzy, and C. G. Fathman.** 1987. Modulation of CD4 by antigenic activation. J Immunol. **138:**1351-4.

93. **Yuwen, H., J. H. Cox, J. Yewdell, J. R. Bennick, and B. Moss.** 1993. Nuclear localization of a double-stranded RNA-binding protein encoded by the vaccinia virus E3L gene. Virology. **195:**732-744.

FIGURE 1

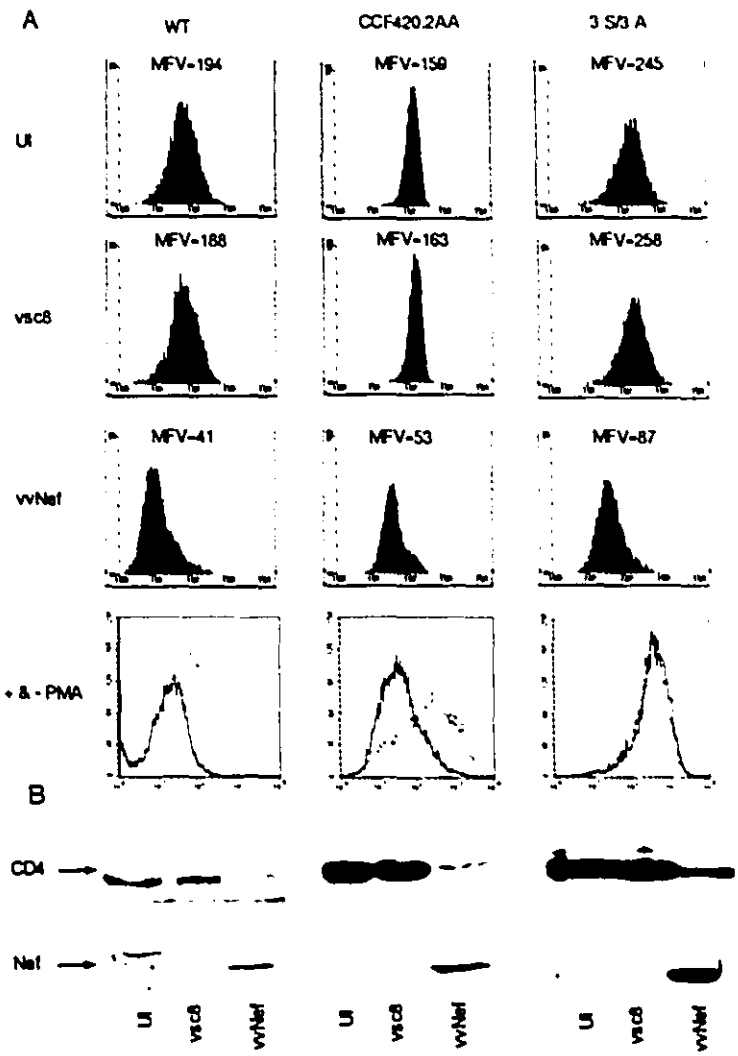
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FIGURE 2

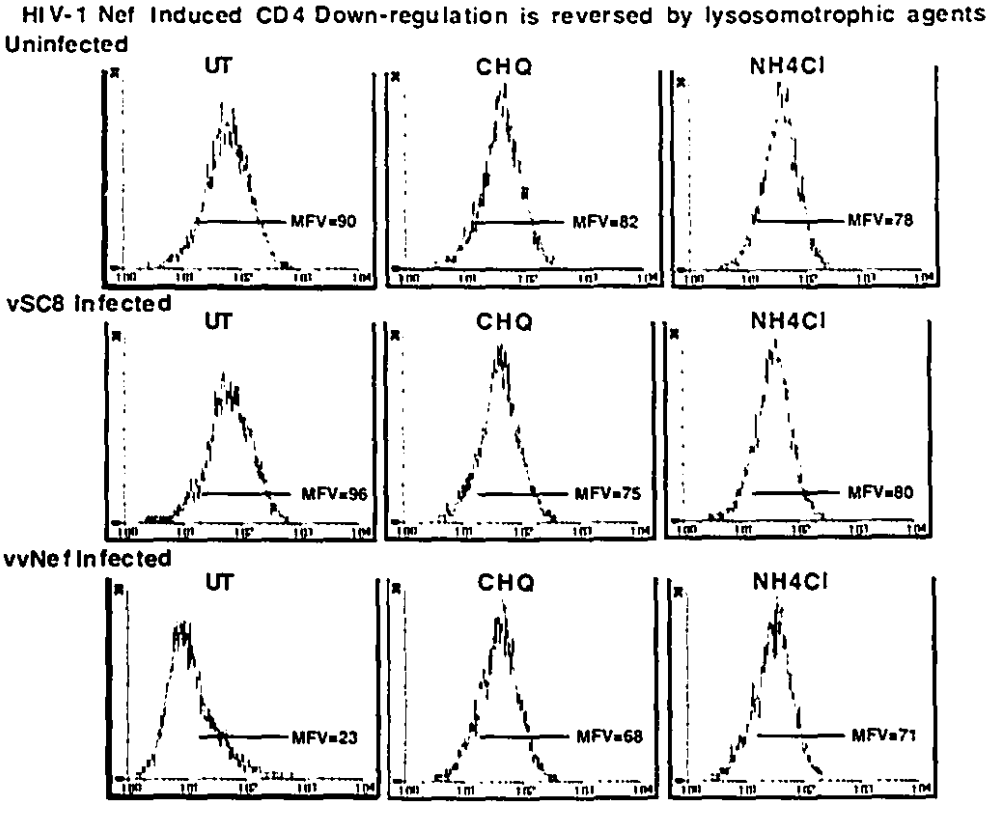
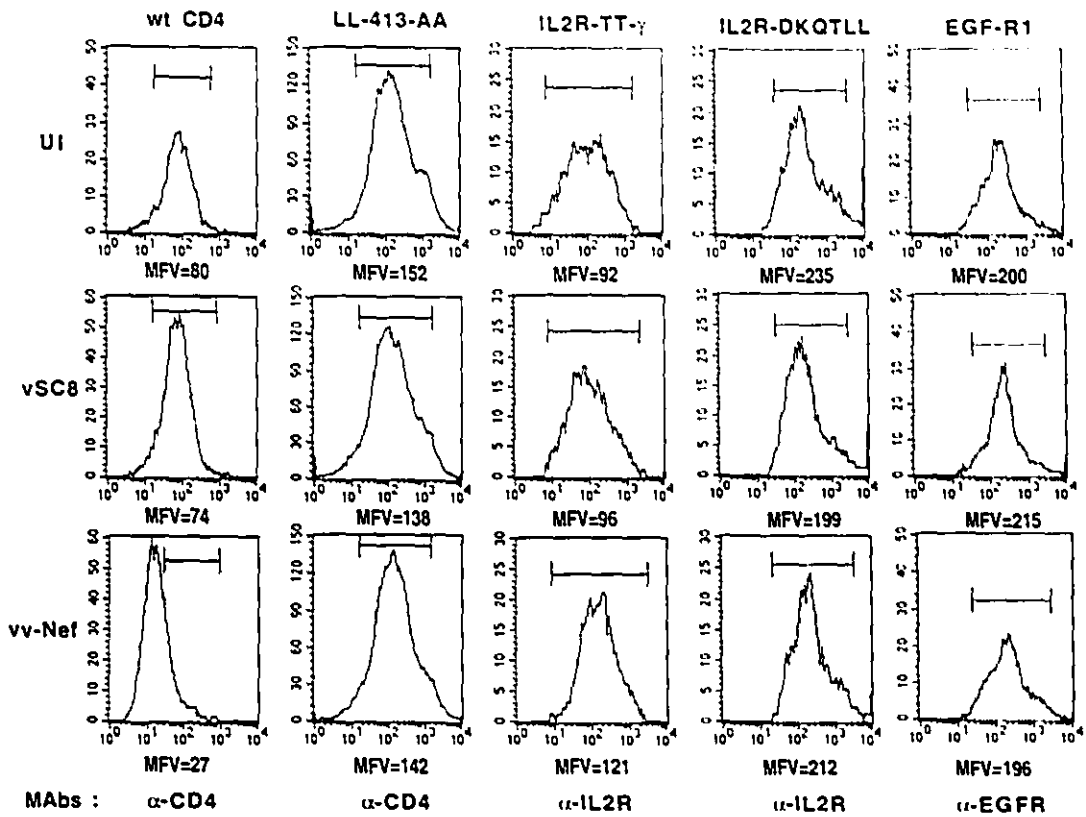

HIV-1 Nef Induced CD4 Down-regulation is reversed by lysosomotrophic agents
Uninfected
UT
CHQ
NH4Cl
MFV=90
MFV=82
MFV=78
vSC8 Infected
UT
CHQ
NH4Cl
MFV=96
MFV=75
MFV=80
vvNef Infected
UT
CHQ
NH4Cl
MFV=23
MFV=68
MFV=71

FIGURE 3A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LL-413-AA
IL2R-TT-γ
IL2R-DKQTLL
EGF-R1
UI
MFV=80
MFV=152
MFV=92
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MFV=200
vSC8
MFV=74
MFV=138
MFV=96
MFV=199
MFV=215
vv-Nef
MFV=27
MFV=142
MFV=121
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MAbs :
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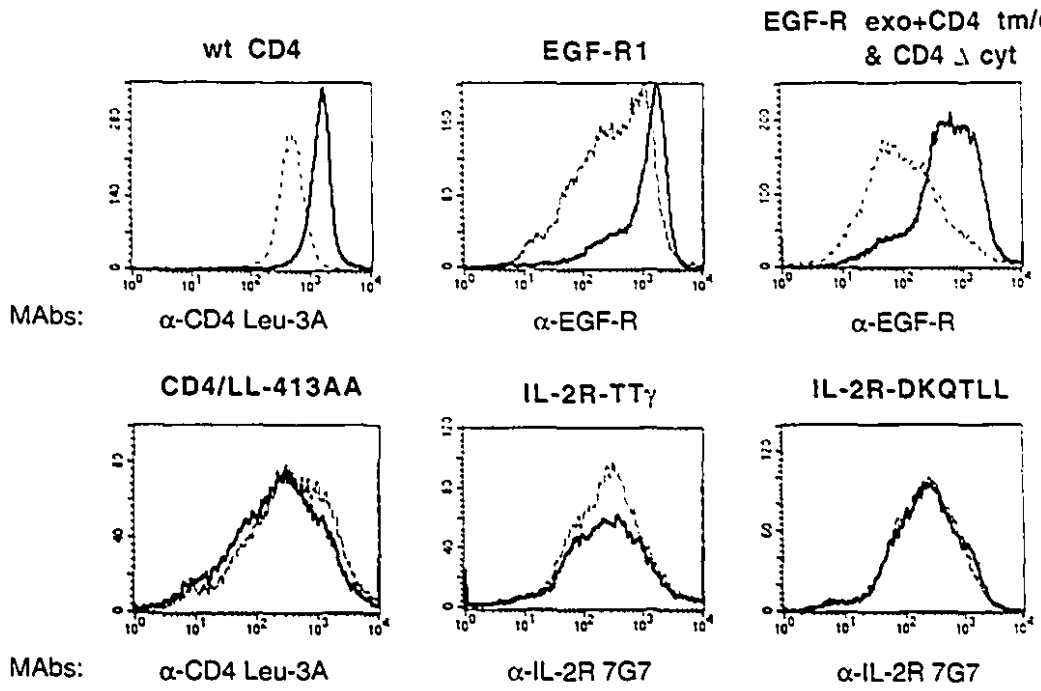
α-CD4
α-IL2R
α-IL2R
α-EGFR

FIGURE 3B


wt CD4
EGF-R1
EGF-R exo+CD4 tm/c & CD4 Δ cyt
MAbs:
α-CD4 Leu-3A
α-EGF-R
α-EGF-R
CD4/LL-413AA
IL-2R-TTγ
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α-IL-2R 7G7

FIGURE 4

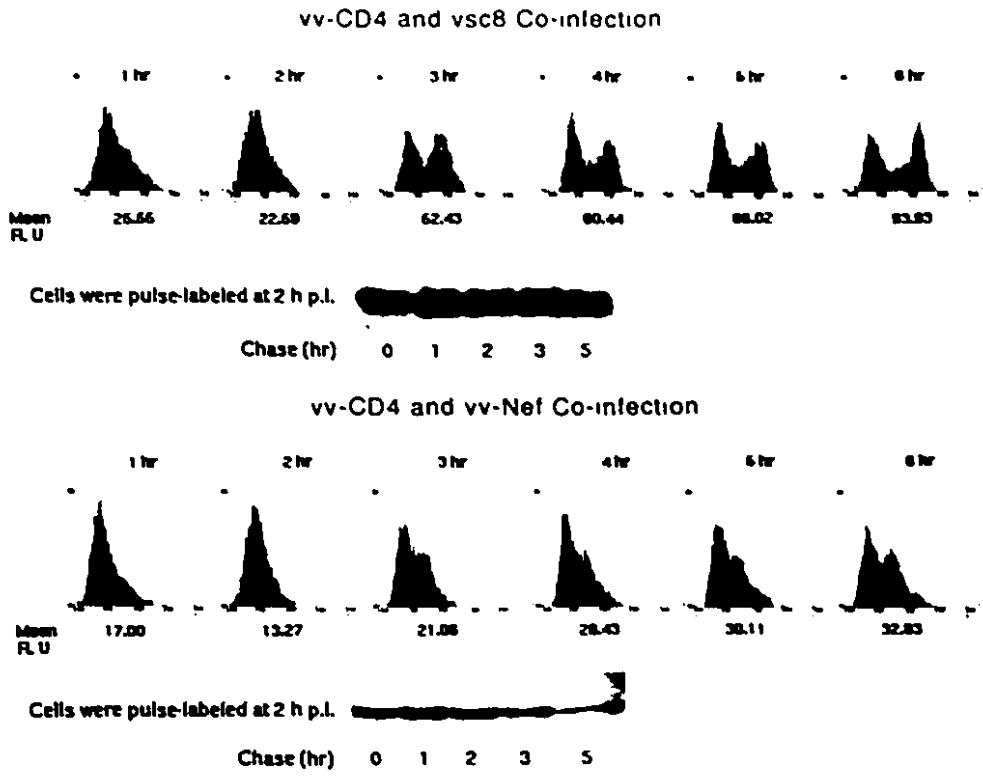

vv-CD4 and vsc8 Co-infection
1 hr
2 hr
3 hr
4 hr
5 hr
6 hr
Mean R.U
26.66
22.69
62.43
80.44
88.02
93.83
Cells were pulse-labeled at 2 h p.i.
Chase (hr) 0 1 2 3 5
vv-CD4 and vv-Nef Co-infection
1 hr
2 hr
3 hr
4 hr
5 hr
6 hr
Mean R.U
17.00
13.27
21.06
28.43
30.11
32.83
Cells were pulse-labeled at 2 h p.i.
Chase (hr) 0 1 2 3 5

FIGURE 5A

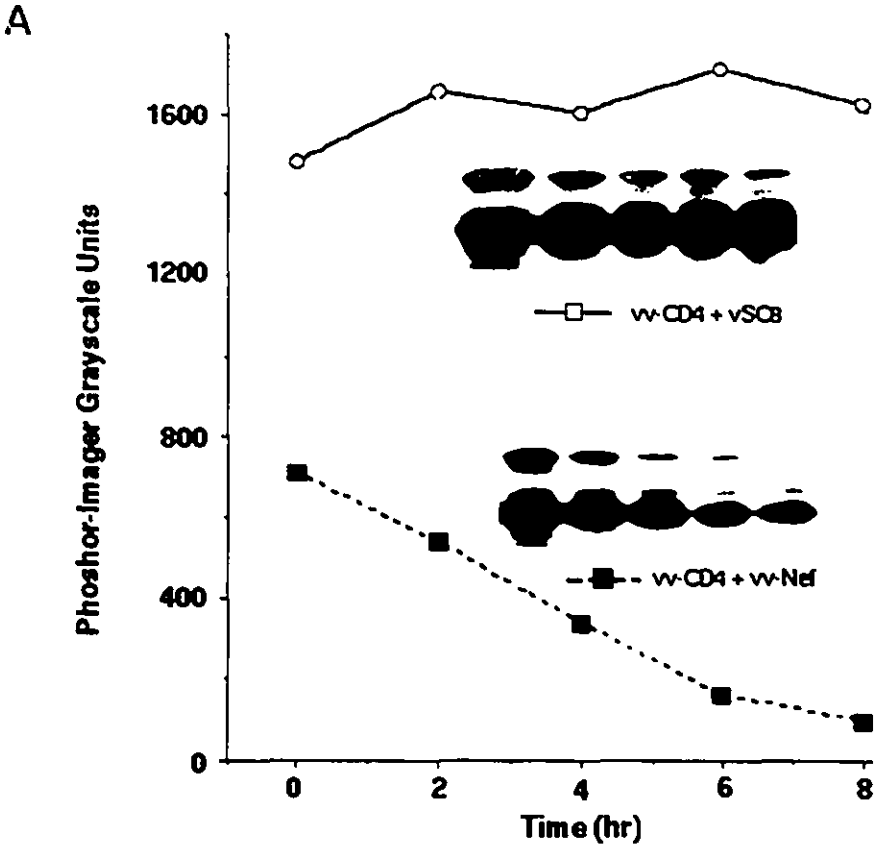

A
1600
1200
800
400
0
Phoshor-Imager Grayscale Units
vv-CD4 + vSC8
vv-CD4 + vv-Nef
0
2
4
6
8
Time (hr)

FIGURE 5B

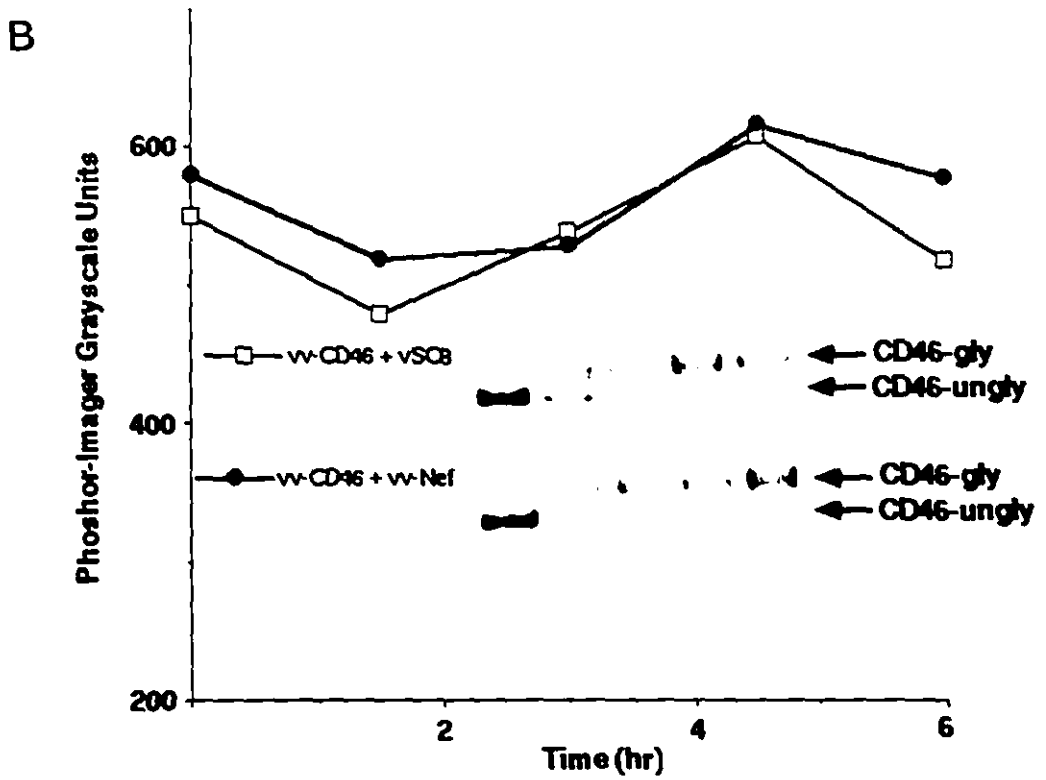

B
Phoshor-Imager Grayscale Units
600
400
200
2
4
6
Time (hr)
vv-CD46 + vSC8
vv-CD46 + vv-Nef
CD46-gly
CD46-ungly
CD46-gly
CD46-ungly

FIGURE 5C

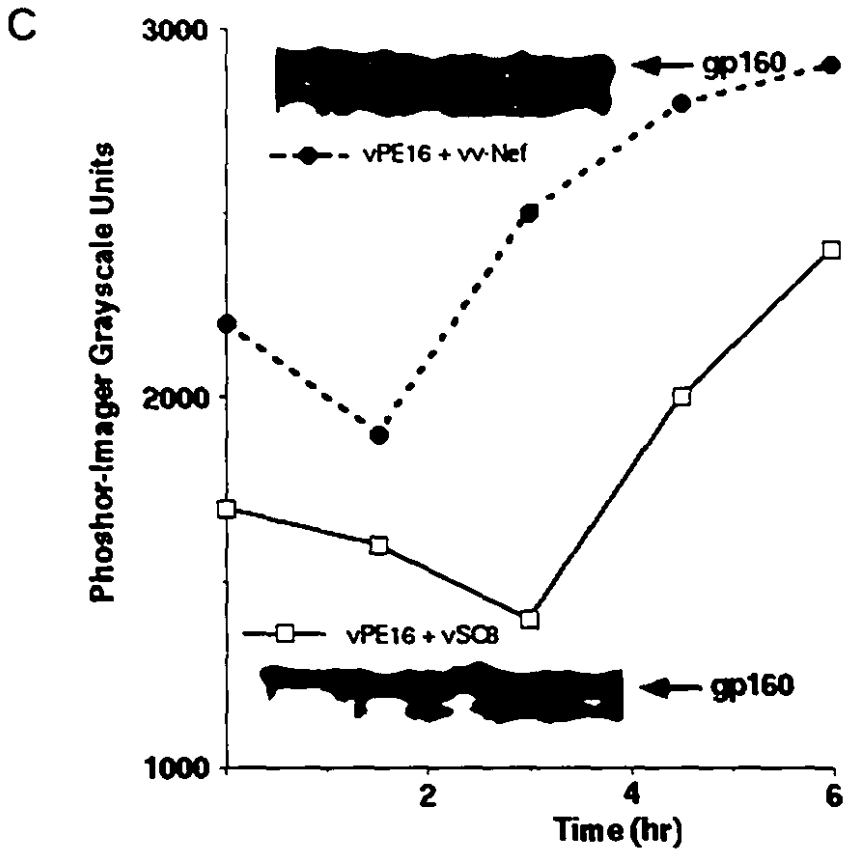

C
3000
2000
1000
Phoshor-Imager Grayscale Units
gp160
vPE16 + vv-Nef
vPE16 + vSC8
gp160
2
4
6
Time (hr)

FIGURE 5D

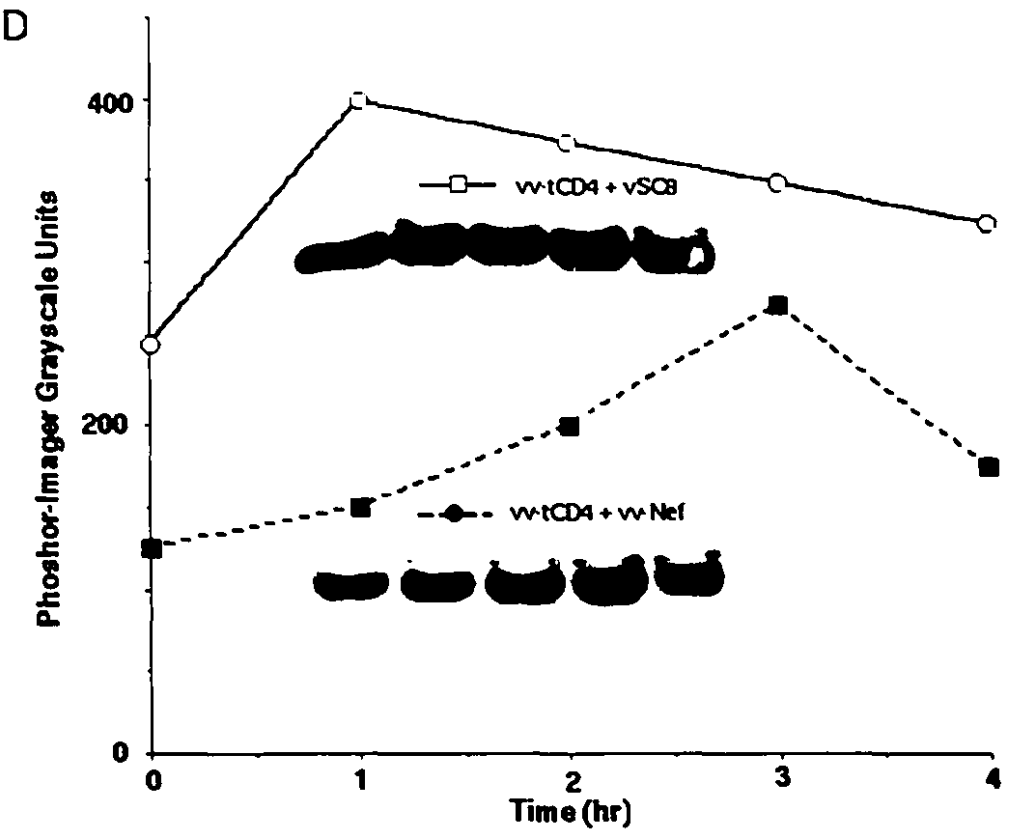

D
400
200
0
Phoshor-Imager Grayscale Units
vv-tCD4 + vSC8
vv-tCD4 + vv-Nef
0
1
2
3
4
Time (hr)

FIGURE 5E

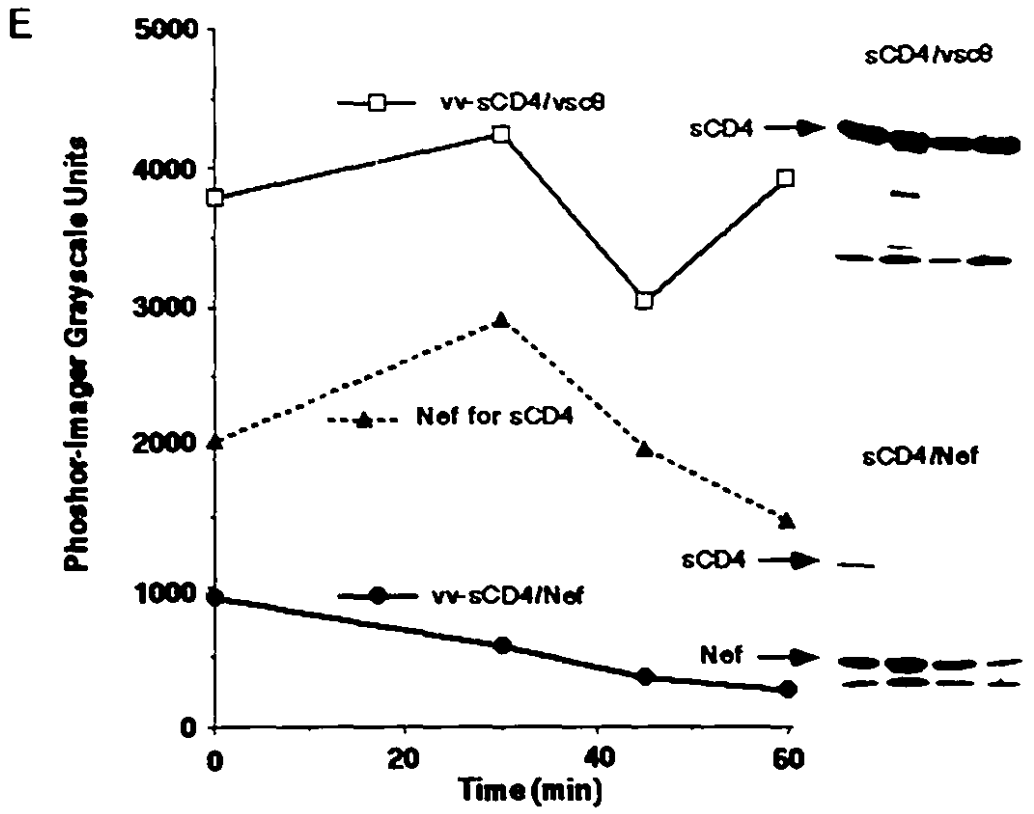

E
5000
4000
3000
2000
1000
0
Phoshor-Imager Grayscale Units
vv-sCD4/vsc8
Nef for sCD4
vv-sCD4/Nef
0
20
40
60
Time (min)
sCD4/vsc8
sCD4
sCD4/Nef
sCD4
Nef

FIGURE 6A

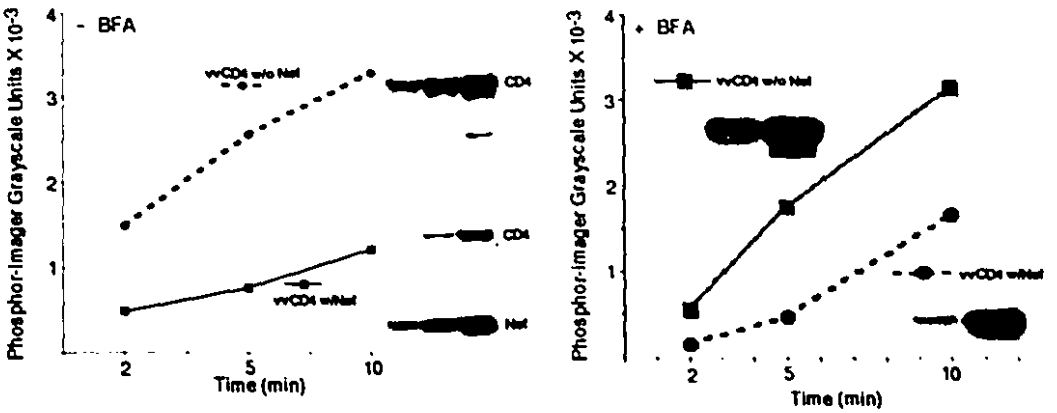

- BFA
Phosphor-Imager Grayscale Units X 10-3
Time (min)
CD4
Nat
+ BFA
Phosphor-Imager Grayscale Units X 10-3
Time (min)

FIGURE 6B

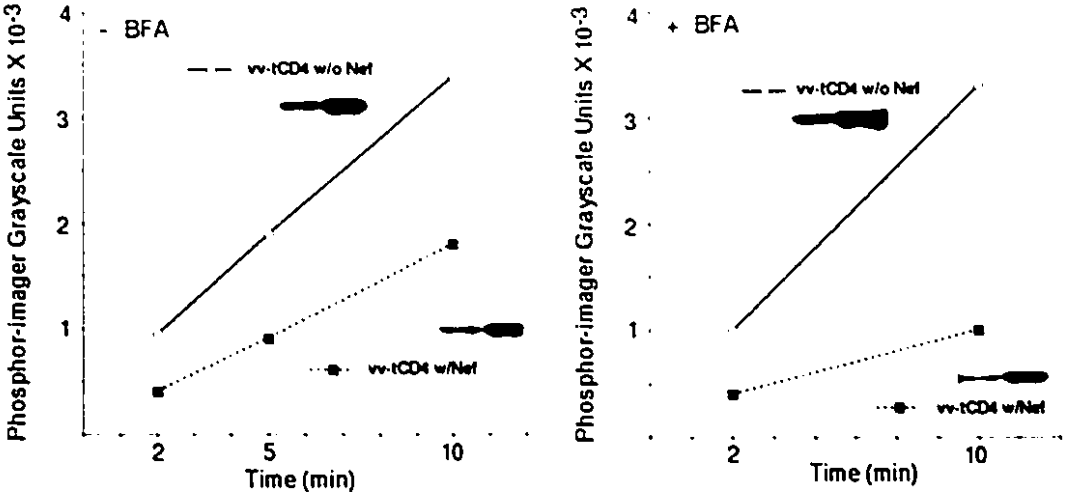
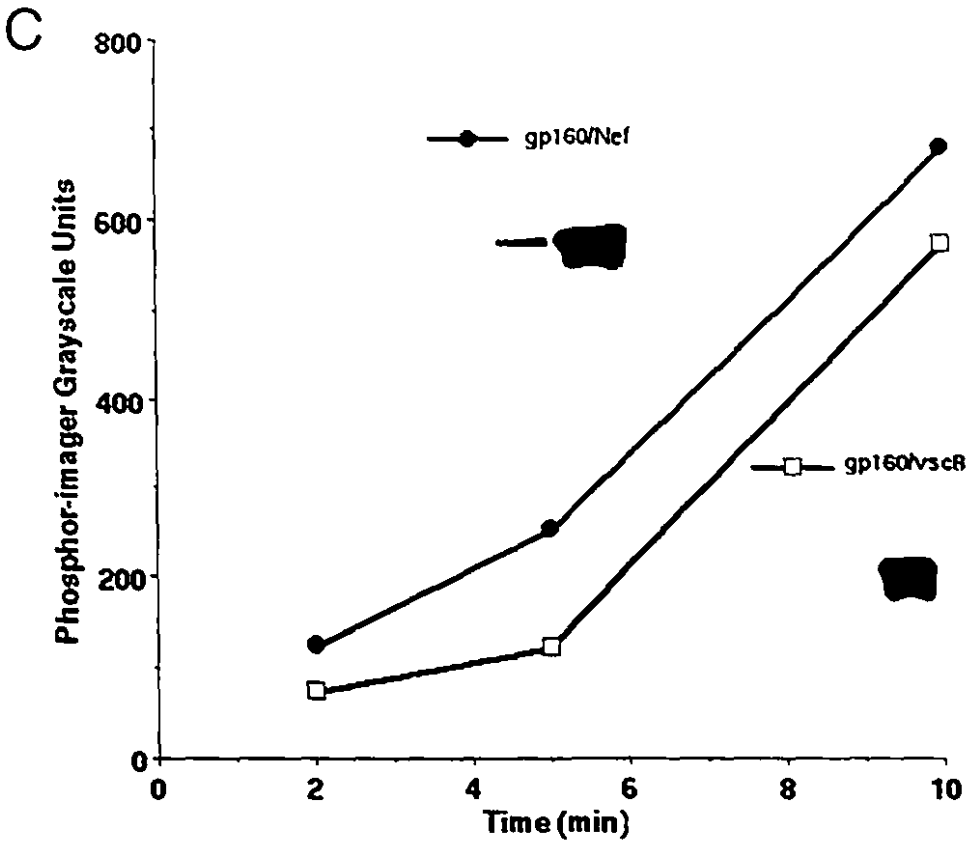

- BFA
vv-tCD4 w/o Nef
vv-tCD4 w/Nef
Phosphor-imager Grayscale Units X 10-3
Time (min)
+ BFA
vv-tCD4 w/o Nef
vv-tCD4 w/Nef
Phosphor-imager Grayscale Units X 10-3
Time (min)

FIGURE 6C


C
800
600
400
200
0
Phosphor-imager Grayscale Units
gp160/Nef
gp160/vsc8
0
2
4
6
8
10
Time (min)

CHAPTER 7

MOLECULAR ANALYSIS OF THE CYTOPLASMIC DOMAIN OF CD4: OVERLAPPING BUT NON-COMPETITIVE REQUIREMENT FOR *lck* ASSOCIATION AND DOWN REGULATION BY NEF

1. Preface

The last chapter described that Nef could down regulate CD4 expression by two mechanisms: from the cell surface and at the level of synthesis of CD4 molecules. In this chapter, we focused our attention on down regulation of cell surface CD4. Using site-directed mutagenesis, we identified the motifs in the cytoplasmic domain of CD4 required for down regulation by Nef. We have also compared the mechanisms by which PMA and Nef down regulate expression of CD4. Finally, since both lck association and susceptibility to Nef require the presence of sequences in the cytoplasmic domain of CD4, we tested the ability of the CD4 mutants to associate with lck.

2. Abstract

Expression of the HIV Nef protein results in the down regulation of cell surface expression of CD4, with a di-leucine motif in the cytoplasmic domain of CD4 being required for this effect. Our results, however, indicate that this di-leucine motif is not sufficient to confer sensitivity to down regulation by Nef. Using site-directed mutagenesis and a transient expression system, we demonstrate that a stretch of amino acids of the cytoplasmic tail of CD4 previously suggested to adopt an helical structure, is also required for the down regulation of CD4 induced by Nef. Some CD4 mutants were found to be resistant to PMA and sensitive to Nef induced down regulation, suggesting that the effects of Nef and PMA implicate interactions with different effector molecules. In addition, our results demonstrate that this motif is also involved in the association of CD4 with the tyrosine kinase p56^{lck}, thus defining a multi-functional domain of CD4. Although there is an overlap in the sequence requirement for *lck* association and susceptibility to Nef, we fail to detect any preferential decrease in *lck* association with CD4 when Nef is expressed during acute HIV infection. Altogether, these results demonstrate that there is an overlapping but non-competitive sequence requirement in the cytoplasmic domain of CD4 for *lck* association and down regulation by Nef.

3. Introduction

The CD4 molecule is a 55 kDa glycoprotein expressed on the surface of a subset of T lymphocytes which recognize antigen presented by Major Histocompatibility Complex (MHC) class II molecules (reviewed in 24). The interaction between CD4 and MHC class II molecules enhances T cell response to antigenic stimulation. CD4 is comprised of four immunoglobulin-like extracellular domains, a single transmembrane domain and a short cytoplasmic tail of 38 residues. It is associated non-covalently with a src-related tyrosine kinase, $p56^{lck}$ (38,52). Mutagenesis studies have demonstrated that two cysteine residues at position 420 and 422 of the cytoplasmic domain of CD4 mediate this interaction (44,51). The CD4/*lck* association is required to bring *lck* in the vicinity of the T cell receptor (TCR) signaling complex and initiate T cell activation upon antigen recognition (16,20). Both antigenic stimulation and PMA treatment induce the down regulation of cell surface expression of CD4 (7,53). This down regulation occurs following a Protein kinase C (PKC)-dependent phosphorylation of three serine residues of the cytoplasmic domain of CD4 (45,46). Following these phosphorylations, *lck* dissociates from CD4; the latter is then internalized and degraded in the lysosomes (21,35,36,48).

Expression of the Human Immunodeficiency virus (HIV) *nef* gene also leads to down regulation of CD4 expression (15). The *nef* gene encodes for a 27 kDa myristylated protein (3,14,18). The function of this protein in viral replication remains controversial as both a positive and a negative effect of Nef *in vitro* have been reported. Early reports suggested that Nef was negatively regulating HIV replication by down regulating transcription from the HIV LTR (1,10,30,34). More recently, a positive effect of Nef on HIV replication in primary peripheral blood lymphocytes (PBL) and macrophages was described (19,27,33,49,58). The presence of Nef increases the infectivity of the progeny virus by a yet unidentified mechanism (11,33,49). Interestingly, the positive effect of Nef is less marked when activated PBLs and T cell lines are infected (13,33,49). *In vivo*, Nef was found to be essential for disease in adult SIV-infected monkeys (26) and for pathogenicity in the SCID-hu model (23). Finally, expression of Nef also affects T cell activation. Again, contradictory results have been reported. Stable cell lines expressing Nef fail to produce Il-2 upon TCR stimulation (31). Transgenic mice expressing Nef in T cells showed either an increase or a decrease in susceptibility to TCR stimulation (8,29,47). Interestingly, Bauer *et al.* showed that Nef has either a stimulatory or an inhibitory effect on T cell activation depending on its subcellular localization (6).

One observation generally agreed upon is the down regulation of cell surface expression of CD4 induced by Nef (4,15,32). Nef induces an accelerated endocytosis of CD4 which is followed by degradation of CD4 in the lysosomal compartment (2,17,37,41,43). This effect of Nef requires the presence of two leucine residues in the cytoplasmic domain of CD4, which are part of a targeting signal for lysosomes (2,40). Our observations suggest, however, that the presence of the two leucine residues is not sufficient to confer susceptibility to down regulation by Nef of other cell surface molecules. To further characterize the residues of CD4 involved in its down regulation by Nef, we have generated a panel of 10 mutants of the cytoplasmic domain of CD4. Our results have enabled us to define a multi-functional domain of the cytoplasmic tail of CD4, involved in nef and PMA down regulation and the association of CD4 with the tyrosine kinase *lck*.

4. Materials and Methods

Cells and plasmids

The A2.01 and Jurkat cell lines were grown in RPMI 1640 medium supplemented with 2mM L-glutamine, 50 mM 2-mercaptoethanol, 50 mg/ml gentamycin and 10% fetal calf serum (FCS). The SVCMV CD4 and mutated CD4 plasmids are described elsewhere (56). The pNLV102 plasmid which encodes the wild type Nef molecule, and pNLV102Xho plasmid encoding for a mutant Nef molecule with a stop codon in its reading frame, were previously described (1).

Transient Transfections

To assess the down regulation of CD4 by Nef, $5X10^6$ A2.01 cells in logarithmic phase were incubated in RPMI medium supplemented with 20% FCS, with a mixture of 20 µg of the SVCMV CD4 or mutated CD4, and 60 µg of either pNLV102 or pNLV102Xho plasmids, for 10 minutes at room temperature. Cells were then electroporated using a Gene pulser unit (Bio-Rad laboratories) at 280 mV and 960 mF. After 10 minutes, cells were washed once and put into culture at 37°C in RPMI medium supplemented as described above. Transfected cells were analyzed 36-40 hours post-transfection. Cells co-transfected with SVCMV CD4 and pNLV102Xho were treated for a further 60 minutes at 37°C with either PMA (Sigma) at 100 ng/ml or with media before analysis. Cells were stained with B66, a CD4 specific monoclonal antibody (mAb) (9), followed by a FITC-conjugated goat anti-mouse antibody (GAM) (Caltag laboratories, San Francisco, CA, USA) and analyzed using a FACscan (Becton Dickinson, Mountain View, CA, USA). Dead cells were gated out using propidium iodide at 2 mg/ml. 5000 live cells were analyzed for each sample. Each mutant was tested in three independent experiments.

Stable transfections

The A2.01 CD4 and C420-2A were described elsewhere (50). The A2.01 S408-15-31A were generated by retroviral infection using the packaging cell line DAMP transfected with the MNC vector as previously described (50). The A2.01 LL413-4A and EMKL cell lines were transfected by electroporation as described above with a combination of 50 µg of the SVCMVCD4 plasmids and 20 µg of the pSV2neo plasmid. Transfected cells were selected in 1 mg/ml G418 (GIBCO). Cells expressing homogenous levels of transfected CD4 molecules were obtained by aseptic sorting using a FACstar Plus (Becton Dickinson).

Western blots

Transfected cells ($5X10^{5}$) were lysed in sample buffer. Proteins were resolved on a 10% SDS-PAGE gel. Proteins were then transferred on nitrocellulose. Membranes were probed using either a rabbit anti-lck serum (a gift from A. Veillette) or a combination of rabbit anti-CD4 serum (a gift from R. Sweet, Smith-Kline & Beechem) and rabbit anti-nef serum. Specific binding of antibodies was determined using a Horse Radish Peroxidase (HRP) conjugated protein A (Amersham, Arlington heights, Ill.) and the ECL reagent kit (Amersham), or [^{125}I]protein A.

Immunoprecipitations

Immunoprecipitations were carried out on 10^{7} A2.01 cells stably transfected with the indicated CD4 mutant and lysed in NP-40 lysis buffer as previously described (50). CD4 or mutant CD4 molecules were immunoprecipitated using protein G sepharose coated with B66 anti-CD4 mAb as previously described (50). Two aliquots equivalent to $5X10^{5}$ cells were taken from each samples to be analyzed by western blot for the presence of total amounts of CD4 and *lck*. Immunoprecipitates were resolved on a 7.5% SDS-PAGE gel. Western blots were performed as described above.

Acute Infection

Viral supernatants were produced in MT-4 cells transfected with either the pNL43 or the pNL43Xho proviral clones (1). Supernatants were collected 60 hours later. Infection of Jurkat cells was performed as follows. $3X10^{6}$ cells were absorbed with equivalent amounts of the viral supernatants as measured by Reverse Transcriptase (28), in 3 ml overnight. The cells were then washed, diluted and put back in culture. The levels of CD4 were monitored daily by cell surface staining and analysis on the FACscan (Becton Dickinson, Mountain View, CA, USA). The percentage of p24 positive cells were also monitored daily by immunofluorescence as previously described (57), and cells were lysed when over 80% of the cells in both pNL43 and pNL43xho samples were positive.

5. Results

Identification of residues in the cytoplasmic domain of CD4 involved in down regulation by Nef

A transient expression system was set up to identify the specific amino acids in the cytoplasmic domain of CD4 which are responsible for down regulation of cell surface expression induced by Nef. As shown in figure 1, transfection of *nef* together with wild type CD4 resulted in a 3 fold decrease in cell surface expression of CD4 as measured by flow cytometry (mean fluorescence value and percentage of positive cells), as compared to cells transfected with the control plasmid (pNLV102Xhc). A decrease in steady state levels of CD4 was observed by western blot analysis under similar conditions (figure 2). The decrease in cell surface expression induced by Nef was comparable in intensity to that induced by PMA treatment (figure 1).

Results illustrated in figure 1 further confirm the requirement for the di-leucine motif in the cytoplasmic tail of CD4 for its down regulation induced by Nef. Western blot analysis further shows the absence of a decrease in steady state levels of this mutant when Nef is co-expressed (figure 2). Interestingly, mutation of only one of the leucines (KL411-3TP) gave us variable results. A slight but reproducible down regulation of this mutant induced by Nef occurred. This down regulation was never to the extent of that observed with wild type CD4 or other mutants. This effect could not be attributed to levels of Nef since Nef expression was monitored in each transfectant by western blot and levels were quite comparable between independent transfections (figure 2B and data not shown). Our previous results from pulse chase experiments indicate that this effect cannot either be attributed to a difference in turn over rate of the CD4 mutants (56).

A number of other molecules such as the Epidermal Growth Factor receptor (EGFR) and the gamma chain of the CD3 complex, also contain a di-leucine motif in their cytoplasmic domain. Our results indicated that these molecules, despite the presence of a functional di-leucine motif, are not down regulated by Nef, suggesting that additional structural constraints exist for the susceptibility of CD4 to be down regulated by Nef (17). We were thus interested in identifying the residues in the cytoplasmic domain of CD4 apart from the two leucines which are required for down regulation by Nef. A series of point mutations located in the minimal region of the cytoplasmic tail of CD4 required in order to get down regulation of expression by Nef were generated.

In particular, we analyzed mutants of a motif previously suggested to confer to the cytoplasmic domain of CD4 an alpha helical structure. This motif has previously been shown to be essential for PMA induced endocytosis and for the Vpu degradation effect (46,56). Mutation of the EMKL sequence to CDTP, leads to a phenotype comparable to that observed following mutation of the di-leucine motif (figure 1 and 2). We have analyzed the mutants previously for prediction of secondary structure of the cytoplasmic domain (56). Interestingly, no alpha helical structure could be modeled for the mutant EMKL. Furthermore, another mutant found to be partially resistant to Nef, the MK407-11P, also could not be modeled as an alpha helix. Hence, the two mutants which could not be modeled as alpha helices were affected in their response to Nef. In contrast, point mutations within the motif (M407P, K411P, EM405-7CD) were still sensitive to Nef and did not significantly disrupt the secondary structure of the cytoplasmic domain of CD4. Mutations EM405-7CD, MQ407-9, C420-2A and S408-15-31A had no effect on down regulation of CD4 by Nef nor on the secondary structure of the cytoplasmic domain of CD4 (table 1). Overall, our results clearly demonstrate the presence in the cytoplasmic tail of CD4 of a structural motif which is responsible for the susceptibility of the CD4 molecule to down regulation by Nef.

Dissociation of sequences in the cytoplasmic domain of CD4 which are required for PMA or Nef induced down regulation

Since PMA and Nef both induce the internalization and lysosomal degradation of CD4, we have tested all of the above described mutants for down regulation induced by PMA. The mutants can be divided into two groups according to their sensitivity to Nef and PMA. In the first group (type 1, 2 and 3 in Table 1), a correlation can be drawn between susceptibility to Nef and to PMA. For example, the EMKL and the LL413-4A mutants are resistant to both Nef and PMA (figure 1 and 2). On the other hand, mutations at positions 405-7-9-20-22 per se do not affect sensitivity of CD4 to Nef and to PMA (table 1).

In the second group (type 4 in Table 1), sensitivity to Nef and PMA could be dissociated (figure 1 and 2). Mutation of the three serine residues (S408-15-31A) involved in CD4 down regulation by PMA did not affect susceptibility of CD4 to Nef, confirming previously published reports. In addition, the K411P mutant was also susceptible to Nef and resistant to PMA (figure 1 and 2). This mutant was also predicted to have an alpha helical secondary structure which includes the di-leucine motif. The

effect of this mutation on sensitivity to PMA thus cannot be attributed to a perturbation of the structure of the cytoplasmic domain of CD4.

These results further demonstrate that the pathways of down regulation of CD4 induced by Nef and PMA are different. In fact, they suggest that both the initiation of internalization which is dependent on phosphorylation of the serines, and later steps of the internalization of CD4 have different requirements in the cytoplasmic domain of CD4, although both Nef and PMA induce degradation of CD4.

Overlapping sequences of the cytoplasmic domain of CD4 are involved in *lck* association and Nef induced down regulation

The mechanism leading to down regulation of CD4 cell surface expression by Nef has yet to be identified. The association of *lck* with CD4 was previously demonstrated to prevent internalization of CD4 (35). An attractive model would be that Nef dissociates *lck* from CD4, thus increasing the rate of CD4 internalization. To verify if the same sequences of CD4 are required for the Nef effect and association with *lck*, A2.01 cells were stably transfected with wild type CD4 or mutants S408-15-31A, C420-2A, LL413-4A and EMKL (figure 3A). To determine the levels of *lck* associated with each mutant, CD4 molecules were immunoprecipitated from total cell lysates with the same antibody used to stain the cell. *lck* association to CD4 was revealed by western blot on the immunoprecipitates (Figure 3B-CD4-associated lck). The presence of *lck* and of the mutant CD4 in the cell lysates were confirmed by western blots (figure 3B-total lck and total CD4). As shown in table 1, Nef could still induce down regulation of cell surface expression and a decrease in steady state levels of the C420-2A mutant, which is not associated with *lck* (figure 3B). This mutant enabled us to clearly dissociate the lck binding motif of CD4 from susceptibility to Nef induced down regulation (type 3 in table 1).

However, two other mutants (LL413-4A and EMKL) which are affected in their response to Nef, had a substantially diminished capacity to associate with *lck* (figure 3B). Overexposure of the membranes revealed a small amount of *lck* associated with LL413-4A and the EMKL mutant (not shown). These results clearly show an overlap in sequences responsible for association of CD4 with *lck* and those required for down regulation of CD4 by Nef.

Nef does not induce dissociation of *lck* from CD4 in acutely HIV infected cells

The above results support the model by which Nef would induce a dissociation of *lck* from CD4 leading to internalization of CD4. To further characterize this model, Jurkat cells which express CD4 and are highly susceptible to infection, were acutely infected with either the HIV clone pNL43, which contains the *nef* sequence used in the above experiments, or pNL43xho which is the isogenic clone in which the Nef protein is absent. Co-pecipitations were performed when 80% of the cells were infected, as indicated by immunofluorescence with an anti-p24 antiboby (data not shown). At this time point, significant amounts of Nef expression could be detected by western blot analysis (figure 4). As expected, Nef induced a down regulation of cell surface expression of CD4 in pNL43 infected cells as compared to cells infected with pNL43Xho (not shown), leading to a 2-3 fold decrease in steady states level of CD4 in the pNL43 infected cells (figure 4). CD4 was immunoprecipitated and an anti-lck immunoblot was performed on the immunoprecipitates. A dramatic decrease in *lck* associated with CD4 was observed in HIV infected cells as compared to uninfected cells (figure 4). Although total *lck* levels were not affected , this decrease was observed in both the pNL43 and pNL43Xho samples, indicating that the effect is not mediated by Nef. More importantly, the decrease in the amount of *lck* co-precipitated with CD4 was proportional (1.5-3 fold) to the decrease in the expression of CD4. We thus could observe the same ratio of *lck* associated to CD4 whether the cells were infected with pNL43 or pNL43Xho. This could be observed at different time points post-infection or at different levels of Nef expression. These results strongly suggest that following infection with HIV, the dissociation of *lck* from CD4 does not contribute to the down regulation of CD4 expression induced by Nef.

6. Discussion

We have characterized the sequence requirements for down regulation of CD4 by Nef or PMA, and those required for the association of CD4 with *lck*. Our results clearly show that in addition to the di-leucine motif, a structure in the cytoplasmic domain of CD4 which is defined by the EMKL sequence confers susceptibility to down regulation by Nef. This motif is not found in other molecules, such as the EGFR and the CD3 g chain, which despite the presence of a functional di-leucine motif are not down regulated by Nef (17). Internalization of CD4 induced by PMA was previously shown to require the presence of a putative alpha helical structure in the cytoplasmic domain of CD4 (46). Interestingly, all the mutants that affect this predicted structure, also affect down regulation induced by Nef. This strongly suggests that a proper conformation of the cytoplasmic domain of CD4 is probably required for exposure of residues directly implicated in down regulation of CD4 by Nef, such as the leucines.

Our mutagenesis data show that different residues of this structure are implicated in the down regulation of CD4 by Nef and PMA. Indeed, mutation of residue 411 resulted in a CD4 molecule which cannot be down regulated by PMA but is still sensitive to Nef, clearly dissociating the two degradative pathways. Mutation of K411 to a proline leaves intact a major portion of the secondary structure of the cytoplasmic domain of CD4. This residue may thus directly interact with a component of the endocytic pathway such as clathrin coated pits, thus targeting CD4 to internalization and lysosomal degradation following PMA treatment. This finding is interesting since several pieces of evidence also implicate the lysosomes in the degradation of CD4 induced by Nef (17,43). It is thus possible that effector molecules other than those involved in PMA induced internalization of CD4, are implicated in internalization of CD4 induced by Nef. Further studies are needed to characterize the internalization and degradation pathway induced by Nef.

As shown in figure 3, both the LL413-4A and the EMKL mutant were severely impaired in their ability to associate with *lck*. These results might explain the different stochiometry of the association of CD4 and CD8 with *lck*; indeed, while more than 40-60% of CD4 molecules are associated with *lck*, only 3-5% of total CD8 molecules coprecipitate with the kinase (54). Although both CD4 and CD8 possess the two cysteine residues critical for association with *lck*, CD8 lacks the EMKL sequence. This suggests that the EMKL sequence may be involved in augmenting the affinity of CD4 for *lck* and is not required for basal interaction between CD4 or CD8 with the tyrosine kinase. These residues could be involved in a direct interaction with *lck*; alternatively, they could

confer to the cytoplasmic tail of CD4 a conformation which would favor its interaction with the tyrosine kinase.

While our results clearly demonstrate that the *lck* binding domain and the Nef susceptibility domain of the cytoplasmic tail of CD4 overlap, mutations of the two cysteine residues implicated in the interaction with *lck* clearly dissociate the two functional domains. Recently, Bandres et al reported that mutations of the two cysteine to serine residues, which abolish association with *lck*, also rendered CD4 resistant to Nef (5). However, it is possible that these added serine residues in the cytoplasmic tail of CD4 could be phosphorylated by the serine kinase associated with Nef (42), thus preventing the down regulation of CD4 induced by Nef.

Results showing the overlap in the structural requirements in the cytoplasmic tail of CD4 for association with *lck* and sensitivity to Nef were recently reported (40). The authors suggested that Nef induced dissociation of *lck* from CD4 to down regulate its expression. In our experiments, acutely infected cells were used to study the effect of Nef on the CD4/*lck* association. Although expression of Nef in the context of HIV does down regulate expression of CD4, our results do not support any preferential dissociation of *lck* from CD4 occurring in cells infected with a virus encoding Nef as compared to cells infected with a virus harboring a non-functional Nef molecule. This observation was made in cells which exhibited either a slight (early) or a marked (late) decrease in cell surface levels of CD4 in the presence of Nef. Interestingly, our results clearly show that the dissociation of *lck* from CD4 in HIV infected cells((25) and figure 4) is not caused by Nef since it was observed in cells infected with a Nef negative virus. It is possible that CD4/*lck* complexes, which are generated very early in the biosynthetic pathway, are degraded following the interaction of CD4 with gp160 or Vpu in the ER (12,22,55).

The discrepancy between our results and those of Salghetti et al can be explained by the differences in the models used. In their report, Nef was not expressed in the context of the virus and the decrease of *lck* association with CD4 was monitored in the absence of any detectable cell surface expressed CD4. In our experiments, co-precipitations were performed when CD4 was still detected at the surface of cells infected with the Nef positive virus, albeit to lower levels than in cells infected with a Nef negative virus. However, since total *lck* levels are not affected in the presence of Nef, we propose that *lck* dissociates from CD4 while CD4 is being degraded in the lysosomes. These results are supported by the report of co-localization of down regulated CD4 with *lck* in an intracellular compartment in Nef transgenic mice (8). These two molecules did not co-localize in non-transgenic litter mates. Taken together, our data completely dissociates PMA induced and Nef induced internalization of CD4. PMA induces dissociation of lck

from CD4, leading to an increase in internalization of CD4 and targeting to lysosomal degradation. Nef also induces internalization and lysosomal degradation of CD4 but of a CD4 still associated with *lck*. However, *lck* is not degraded, thus ultimately resulting in an increase in the amount of *lck* not associated with CD4.

The results presented here also have some implications on the possible mechanisms by which Nef exerts a positive effect on HIV replication. We have previously demonstrated that the CD4/*lck* association down regulates HIV replication (50). Although a positive effect of Nef can be impaired by mutation of the proline rich regions without affecting CD4 down regulation (39), it is still highly conceivable that down regulation of the CD4/*lck* association from the cell surface induced by Nef contributes to the positive effect of Nef. Indeed, down regulation of CD4/*lck* from the cell surface by Nef could abolish the negative signal for HIV replication generated through CD4/*lck* and lead to increased HIV replication. Testing of this hypothesis is under way.

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7. References

1. **Ahmad, N. and S. Venkatesan. 1988.** Nef protein of HIV-1 is a transcriptional repressor of HIV-1 LTR . Science **241**:1481-1485.

2. **Aiken, C., J. Konner, N. R. Landau, M. E. Lenburg, and D. Trono.** 1994. Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. Cell **76**:853-864.

3. **Allan, J. S., J. E. Coligan, T. H. Lee, M. F. McLane, P. J. Kaniki, J. E. Groopman, and M. Essex.** 1985. A new HTLV-III/LAV encoded antigen detected by antibodies from AIDS patients. Science **230**:810-813.

4. **Anderson, S., D. C. Shugars, R. Swanstrom, and J. V. Garcia.** 1993. Nef from primary isolates of human immunodeficiency virus type 1 suppresses surface CD4 expression in human and mouse T cells. J. Virol. **67**:4923-4931.

5. **Bandres, J. C., A. S. Shaw, and L. Ratner.** 1995. HIV-1 Nef protein downregulation of CD4 surface expression: relevance of the lck binding domain of CD4. Virology **207**:338-341.

6. **Bauer, A. S., E. T. Sawai, P. Dazin, W. J. Fantl, C. Cheng-Mayer, and B. M. Peterlin.** 1994. HIV-1 nef leads to inhibition or activation of T cells depending on its intracellular localization. Immunity **1**:373-384.

7. **Blue, M.-L., D. A. Hafler, K. A. Craig, H. Levine, and S. F. Schlossman.** 1987. Phosphorylation of CD4 and CD8 molecules following T cell triggering. J. Immunol. **139**:3949-3954.

8. **Brady, H. J., D. J. Pennington, C. G. Miles, and E. A. Dzierzak.** 1993. CD4 cell surface downregulation in HIV-1 Nef transgenic mice is a consequence of intracellular sequestration. EMBO J. **12**:4923-4932.

9. **Carrel, S., A. Moretta, G. Pantaleo, G. Tambussi, P. Isler, B. Perussia, and J. C. Cerottini.** 1988. Stimulation and proliferation of CD4+ peripheral blood T lymphocytes induced by an anti-CD4 monoclonal antibody. Eur. J. Immunol. **18**:333-339.

10. **Cheng-Mayer, C., P. Iannello, K. Shaw, P. A. Luciw, and J. A. Levy.** 1989. Differential effects of nef on HIV replication: implications for viral pathogenesis in the host. Science **246:**1629-1632.

11. **Chowers, M. Y., C. A. Spina, T. J. Kwoh, N. J. Fitch, D. D. Richman, and J. C. Guatelli.** 1994. Optimal infectivity in vitro of human immunodeficiency virus type 1 requires an intact nef gene. J. Virol. **68:**2906-2914.

12. **Crise, B., L. Buonocore, and J. K. Rose.** 1990. CD4 is retained in the endoplasmic reticulum by the human immunodeficiency virus type 1 glycoprotein precursor. J. Virol. **64:**5585-5593.

13. **de Ronde, A., B. Klaver, W. Keulen, L. Smit, and J. Goudsmit.** 1992. Natural HIV-1 NEF accelerates virus replication in primary human lymphocytes. Virology **188:**391-395.

14. **Franchini, G., G. M. Robert, J. Ghrayeb, N. T. Chang, and F. Wong-Staal.** 1986. Cytoplasmic localization of the 3`orf protein in cultured T cells. Virology **155:**593-599.

15. **Garcia, J. V. and A. D. Miller.** 1991. Serine phosphorylation-independent downregulation of cell-surface CD4 by nef. Nature **350:**508-511.

16. **Glaichenhaus, N., N. Shastri, D. R. Littman, and J. M. Turner.** 1991. Requirement for association of p56lck with CD4 in antigen-specific signal transduction in T cells. Cell **64:**511-520.

17. **Gratton, S., S. Popov, S. Chandrasekhar, C. Broder, R.-P. Sekaly, and S. Venkatesan.** EMBO J. Submitted for publication.

18. **Guy, B., M. P. Kieny, Y. Riviere, C. LePeuch, K. Dott, M. Girard, L. Montagnier, and J. P. Lecocq.** 1987. HIV F/3` orf encodes a phosphorylated GTP-binding protein resembling an oncogene product. Nature **330:**266-269.

19. **Hammes, S. R., E. P. Dixon, M. H. Malim, B. R. Cullen, and W. C. Greene.** 1989. Nef protein of human immunodeficiency virus type 1: evidence against its role as a transcriptional inhibitor. Proc. Natl. Acad. Sci. USA **86**:9549-9553.

20. **Haughn, L., S. Gratton, L. Caron, R. P. Sekaly, A. Veillette, and M. Julius.** 1992. Association of tyrosine kinase p56lck with CD4 inhibits the induction of growth through the alpha beta T - cell receptor. Nature **358**:328-331.

21. **Hurley, T. R., K. Luo, and B. M. Sefton.** 1989. Activators of protein kinase C induce dissociation of CD4, but not CD8, from p56lck. Science **245**:407-409.

22. **Jabbar, M. A. and D. P. Nayak.** 1990. Intracellular interaction of human immunodeficiency virus type 1 (ARV-2) envelope glycoprotein gp160 with CD4 blocks the movement and maturation of CD4 to the plasma membrane. J. Virol. **64**:6297-6304.

23. **Jamieson, B. D., G. M. Aldrovandi, V. Planelles, J. B. Jowett, L. Gao, L. M. Bloch, I. S. Chen, and J. A. Zack.** 1994. Requirement of human immunodeficiency virus type 1 nef for in vivo replication and pathogenicity. J. Virol. **68**:3478-3485.

24. **Janeway, C. A. Jr.** 1992. The T cell receptor as a multicomponent signalling machine: CD4/CD8 coreceptors and CD45 in T cell activation. Ann. Rev. Immunol. **10**:645-675.

25. **Kanner, S. B. and O. K. Haffar.** 1995. HIV-1 down regulates CD4 costimulation of TcR/CD3 directed tyrosine phosphorylation through CD4/p56lck dissociation. J. Immunol. **154**:2996-3005.

26. **Kestler, H. W., 3d, D. J. Ringler, K. Mori, D. L. Panicali, P. K. Sehgal, M. D. Daniel, and R. C. Desrosiers.** 1991. Importance of the nef gene for maintenance of high virus loads and for development of AIDS. Cell **65**:651-662.

27. **Kim, S., K. Ikeuchi, R. Byrn, J. Groopman, and D. Baltimore.** 1989. Lack of a negative influence on viral growth by the nef gene of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **86**:9544-9548.

28. **Lee, M. H., K. Sano, F. E. Morales, and D. T. Imagawa.** 1987. Sensitive reverse transcriptase assay to detect and quantitate human immunodeficiency virus. J. Clin. Mirobiol. **25**:1717-1721.

29. **Lindemann, D., R. Wilhelm, P. Renard, A. Althage, R. Zinkernagel, and J. Mous.** 1994. Severe immunodeficiency associated with a human immunodeficiency virus 1 NEF/3'-long terminal repeat transgene. J. Exp. Med. **179**:797-807.

30. **Luciw, P. A., C. Cheng-Mayer, and J. A. Levy.** 1987. Mutational analysis of the human immunodeficiency virus: the orf-B region down regulates virus replication. Proc. Natl. Acad. Sci. USA **84**:1434-1438.

31. **Luria, S., I. Chambers, and P. Berg.** 1991. Expression of the type 1 human immunodeficiency virus Nef protein in T cells prevents antigen receptor-mediated induction of interleukin 2 mRNA. Proc. Natl. Acad. Sci. USA **88**:5326-5330.

32. **Mariani, R. and J. Skowronski.** 1993. CD4 down-regulation by nef alleles isolated from human immunodeficiency virus type 1-infected individuals. Proc. Natl. Acad. Sci. USA **90**:5549-5553.

33. **Miller, M. D., M. T. Warmerdam, I. Gaston, W. C. Greene, and M. B. Feinberg.** 1994. The human immunodeficiency virus-1 nef gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages. J. Exp. Med. **179**:101-113.

34. **Niederman, T. M., B. J. Thielan, and L. Ratner.** 1989. Human immunodeficiency virus type 1 negative factor is a transcriptional silencer. Proc. Natl. Acad. Sci. USA **86**:1128-1132.

35. **Pelchen-Matthews, A., I. Boulet, D. R. Littman, R. Fagard, and M. Marsh.** 1992. The protein tyrosine kinase p56lck inhibits CD4 endocytosis by preventing entry of CD4 into coated pits. J. Cell Biol. **117**:279-290.

36. **Pelchen-Matthews, A., I. J. Parsons, and M. Marsh.** 1993. Phorbol ester-induced downregulation of CD4 is a multistep process involving dissociation from p56 lck,

increased association with clathrin-coated pits and altered endosomal sorting. J. Exp. Med. **178**:1209-1222.

37. **Rhee, S. S. and J. W. Marsh.** 1994. HIV-1 Nef activity in murine T cells. CD4 modulation and positive enhancement. J. Immunol. **152**:5128-5134.

38. **Rudd, C. E., J. M. Trevillyan, J. V. Dasgupta, L. L. Wong, and S. F. Schlossman.** 1988. The CD4 antigen is complexed in detergent lysates tom protein tyrosin kinase (pp58) from human T lymphocytes. Proc. Natl. Acad. Sci. USA **85**:5190

39. **Saksela, K., G. Cheng, and D. Baltimore.** 1995. Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef+ viruses but not for down-regulation of CD4. EMBO J. **14**:484-491.

40. **Salghetti, S., R. Mariani, and J. Skowronski.** 1995. Human immunodeficiency virus type 1 Nef and p56lck protein-tyrosine kinase interact with a common element in CD4 cytoplasmic tail. Proc. Natl. Acad. Sci. USA **92**:349-353.

41. **Sanfridson, A., B. R. Cullen, and C. Doyle.** 1994. The simian immunodeficiency virus Nef protein promotes degradation of CD4 in human T cells. J. Biol. Chem. **269**:3917-3920.

42. **Sawai, E. T., A. Baur, H. Struble, B. M. Peterlin, J. A. Levy, and C. Cheng-Mayer.** 1994. Human immunodeficiency virus type 1 Nef associates with a cellular serine kinase in T lymphocytes. Proc. Natl. Acad. Sci. USA **91**:1539-1543.

43. **Schwartz, O., A. Dautry-Varsat, B. Goud, V. Marechal, A. Subtil, J. M. Heard, and O. Danos.** 1995. Human immunodeficiency virus type 1 Nef induces accumulation of CD4 in early endosomes. J. Virol. **69**:528-533.

44. **Shaw, A. S., J. Chalupny, A. Whitney, C. Hammond, K. E. Amrein, P. Kavathas, B. M. Sefton, and J. K. Rose.** 1990. Short related sequences in the cytoplasmic domains of CD4 and CD8 mediated binding to the amino-terminal domain of the p56lck tyrosine protein kinase. Mol. Cell. Biol. **10**:1853-1862.

45. **Shin, J., C. Doyle, Z. Yang, D. Kappes, and J. Strominger.** 1990. Structural features of the cytoplasmic region of CD4 required for internalization. EMBO J. **9**:425-434.

46. **Shin, J., R. L. J. Dunbrack, S. Lee, and J. L. Strominger.** 1991. Phosphorylation-dependent down-modulation of CD4 requires a specific structure within the cytoplasmic domain of CD4. J. Biol. Chem. **266**:10658-10665.

47. **Skowronski, J., D. Parks, and R. Mariani.** 1993. Altered T cell activation and development in transgenic mice expressing the HIV-1 nef gene. EMBO J. **12**:703-713.

48. **Sleckman, B. P., J. Shin, V. E. Igras, T. L. Collins, J. L. Strominger, and S. J. Burakoff.** 1992. Disruption of the CD4-p56lck complex is required for rapid internalization of CD4. Proc. Natl. Acad. Sci. USA **89**:7566-7570.

49. **Spina, C. A., T. J. Kwoh, M. Y. Chowers, J. C. Guatelli, and D. D. Richman.** 1994. The importance of nef in the induction of human immunodeficiency virus type 1 replication from primary quiescent CD4 lymphocytes. J. Exp. Med. **179**:115-123.

50. **Tremblay, M., S. Meloche, S. Gratton, M. A. Wainberg, and R. P. Sekaly.** 1994. Association of p56lck with the cytoplasmic domain of CD4 modulates HIV-1 expression. EMBO J. **13**:774-783.

51. **Turner, J. M., M. H. Brodsky, B. A. Irving, S. D. Levin, R. M. Perlmutter, and D. R. Littman.** 1990. Interaction of the unique N-terminal region of tyrosine kinase p56lck with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. Cell **60**:755-765.

52. **Veillette, A., M. A. Bookman, E. M. Horack, and J. B. Bolen.** 1988. The CD4 and the CD8 T Cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56ick. Cell **55**:301-308.

53. **Weyand, C. M., J. Goronzy, and G. Fathman.** 1987. Modulation of CD4by antigenic activation. J. Immunol **138**:1351-1354.

54. **Wiest, D. L., L. Yuan, J. Jefferson, P. Benveniste, M. Tsokos, R. D. Klausner, L. H. Glimcher, L. E. Samelson, and A. Singer.** 1993. Regulation of T cell receptor expression in immature CD4+CD8+ thymocytes by p56lck tyrosine kinase:basis for differential signaling by CD4 and CD8 in immature thymocytes expressing both coreceptor molecules. J. Exp. Med. **178:**1701-1712.

55. **Willey, R. L. ., F. Maldarelli, M. A. Martin, and K. Strebel.** 1992. Human immunodeficiency virus type 1 Vpu protein induces rapid degradation of CD4. J. Virol. **68:**7193-7200.

56. **Yao, X.-J., J. Friborg, F. Checroune, S. Gratton, F. Boisvert, R.-P. Sekaly, and E. A. Cohen.** 1995. Degradation of CD4 induced by human immunodeficiency virus type 1 vpu protein: a predicted alpha-helical structure in the proximal cytoplasmic region of CD4 contributes to vpu sensitivity. Virology, in press.

57. **Yao, X.-J., S. Garzon, F. Boisvert, W. A. Haseltine, and E. A. Cohen.** 1993. The effect of vpu on HIV-1 induced syncythia formation. J. AIDS **6:**135-141.

58. **Zazopoulos, E. and W. A. Haseltine.** 1992. Mutational analysis of the human immunodeficiency virus type 1 Eli Nef function. Proc. Natl. Acad. Sci. USA **89:**6634-6638.

Legends

Figure 1. Cell surface expression of CD4 and CD4 mutants in the presence (nef) or absence of Nef (xho), or after PMA treatment (xho/PMA). Transiently transfected cells were stained with anti-CD4 B66 mAb followed by a FITC-conjugated GAM. Results are illustrated on a four decade logarithmic scale. For each sample, a negative control consisting of staining with the FITC-conjugated GAM alone was done. Markers were set for each sample on the negative control such that 1% of the cells were right of the marker. MFV, that is the mean fluorescence value of cells on the right of the marker, and percentage of positive cells are indicated for each histograms.

Figure 2. Steady state levels of CD4 and CD4 mutants in cells co-transfected with Nef (+) or the negative control (-). CD4 expression (Panel A) was detected with an anti-CD4 serum and Nef expression (Panel B) was detected using an anti-Nef serum. Hybridized proteins were revealed using HRP-conjugated protein A (Amersham) and the ECL reagent kit (Amersham).

Figure 3. Association of $p56^{lck}$ with wild type CD4 and mutant CD4 molecules. A) Cell surface expression of stable transfectants of the A2.01 cell line. Cells were stained using the B66 anti-CD4 mAb followed by a FITC-coupled GAM, and analyzed on a FACScan. B) *lck* association with mutated CD4 and wild type CD4. Cells stably transfected with either S408-15-31A (lane 1), EMKL (lane 2), LL413-4A (lane 3), C420-2A (lane 4), and wild type CD4 (lane 5) were lysed in NP-40 lysis buffer. CD4 was immunoprecipitated using protein G sepharose beads coated with B66 anti-CD4 mAb. Immunoprecipitates (CD4-associated lck) or cell lysates (total lck and total CD4) were resolved on a 7.5% SDS-Page and proteins were transferred on nitrocellulose. Membranes were hybridized using either an anti-lck serum or an anti-CD4 serum as indicated, followed by [^{125}I]protein A.

Figure 4 Analysis of the association of *lck* with CD4 in cells acutely infected with either pNL43 or pNLXho. Two independent experiments are shown. An equal number of live acutely infected cells or uninfected control cells were lysed in NP-40 lysis buffer and CD4 was immunoprecipitated as in Figure 3. The immunoprecipitates (CD4-associated lck) and a portion of the cell lysates (total CD4, total lck and Nef) were resolved on SDS-PAGE and proteins were transferred on nitrocellulose. Membranes were hybridized using either an anti-lck serum or an anti-CD4 serum or an anti-Nef serum as indicated, followed

by [^{125}I]protein A. The membranes were exposed to Kodak storage phosphor screens and quantification of specific bands was done on PhosphorImager devised by ImageQuant Software Package (Molecular Dynamics, Sunnyvale,CA).

Table 1. Summary of the mutagenesis results[a].

CD4 MUTANTS	DOWNREGULATION BY NEF	DOWNREGULATION BY PMA	ASSOCIATION WITH *lck*
Type 1			
CD4	+	+	+
EM 405-7CD	+	+	n.d.
MQ 407-9CD	+	+	n.d.
M407P	+	+	n.d.
Type 2			
LL413-4A	-	-	-
EMKL[b]	-	-	-
KL411-31P	+/-	-	n.d.
MK407-11P	+/-	-	n.d.
Type 3			
C420-2A	+	+	-
Type 4			
S408-15-31A	+	-	+
K411P	+	-	n.d.

[a]A + equals to wild type phenotype, a - corresponds to a complete loss of wild type phenotype. In the case of nef downregulation, +/- reflects a shift in CD4 expression in the presence of nef which is intermediate between wild type and resistant mutants. n.d. is not determined.

[b]The substitutions for the EMKL motif are EMKL405-7-11-13CDTP.

FIGURE 1

A)

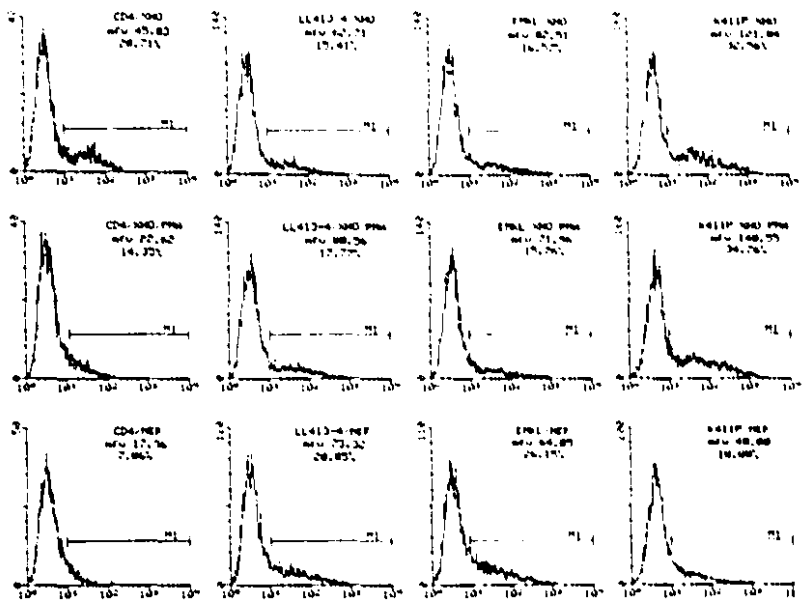


FIGURE 2

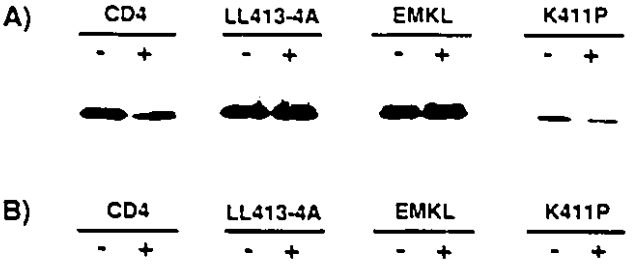


FIGURE 3 A)

A)

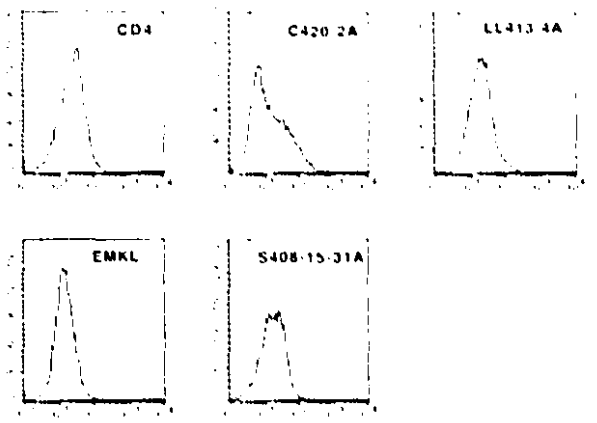


FIGURE 3 B)

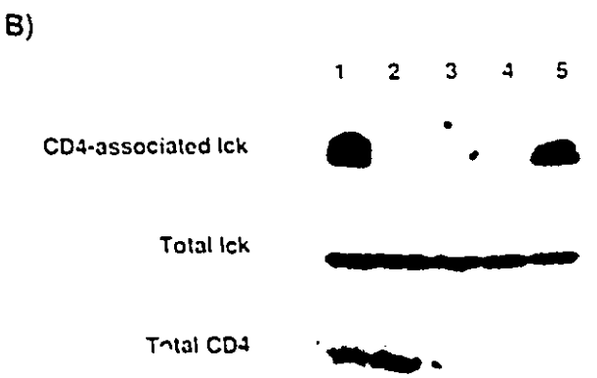
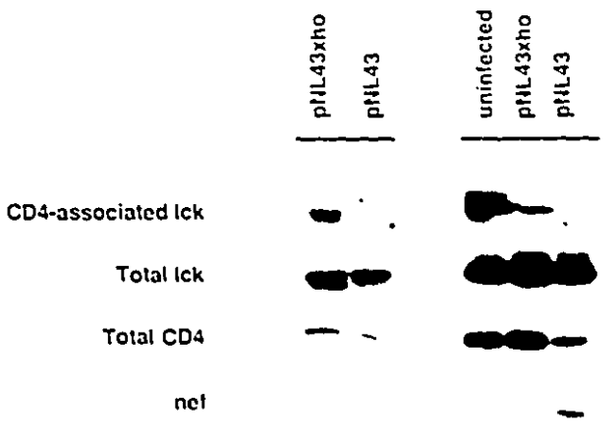


FIGURE 4


pNL43xho
pNL43
uninfected
pNL43xho
pNL43
CD4-associated lck
Total lck
Total CD4
nef

CHAPTER 8: GENERAL DISCUSSION

Infection by HIV results in a general state of immunodeficiency which leaves the host unable to defend himself against invasion by other organisms such as viruses and bacteria, and against propagation of tumor cells. It appears that death of the host occurs not as a direct consequence of HIV infection but from complications arising from these opportunistic diseases.

The understanding of the pathogenesis of HIV relies on a better knowledge of the effect of the virus on the immune system. CD4 positive helper T cells are the primary host cells for HIV infection since the CD4 molecule is the receptor for the virus. These helper cells play a crucial regulating role of the host immune response against foreign aggressions. Upon recognition of their cognate antigen, they start secreting cytokines which stimulate the generation and activation of cytotoxic T cells, and promote the differentiation and amplification of B cells.

In vitro studies have demonstrated that infection of T cells by HIV requires their activation. Indeed, T cell division must occur for proviral DNA integration into the cellular chromosomes. In addition, cellular transcription factors such as NF-κB, which are required for efficient transcription from the HIV LTR are only found in activated T cells where they are normally implicated in directing transcription from the IL-2 promoter. Previous studies have demonstrated that signals generated through CD4 can either enhance or inhibit T cell activation. Considering that some transcription factors are implicated both in regulating HIV and IL-2 gene transcription, it is reasonable to assume that signals generated through CD4 will also modulate HIV replication. It is thus imperative to fully elucidate the physiological role of the HIV receptor in generating activation signals to better understand the HIV life cycle.

In an effort to undercover the interrelationship between host and viral proteins, we here present evidence that HIV has evolved to share activation signals with its host and even modulate these signals for its own purpose. The discussion will thus first focus on the function of CD4 in normal T cell activation, and thereafter will elaborate on how signals generated through CD4 modulate and are modulated by HIV proteins.

SIGNALS GENERATED THROUGH CD4 REGULATE T CELL ACTIVATION

1. What is the role of the association of lck with CD4 in regulating its tyrosine kinase activity?

As described in the Introduction, the tyrosine kinase lck is required to initiate T cell activation. Indeed, lck is directly involved in phosphorylation of the ζ chain and the recruitment and phosphorylation of the ZAP tyrosine kinase, which are the earliest detectable events following TcR ligation by antigen or anti-TcR Abs.

Between 50% to 80% of the total lck is associated with CD4, suggesting that this interaction is important for the regulation of this kinase. Two non-exclusive models can be proposed to define the mechanism by which CD4 regulates lck activity. First, association of lck with CD4 would be required to transmit extracellular signals and activate its tyrosine kinase activity. Second, CD4 would regulate lck activity by controlling its subcellular localization.

1) The concept that the CD4/lck complex can transmit extracellular signals came from studies which demonstrated that cross-linking of CD4 with Ab leads to an increase in tyrosine kinase activity of the CD4-associated lck (36,62). As shown in chapter 4, the extracellular domain of CD4 is not required to activate lck, as the lck associated with the EGFRCD4 chimera can be activated by oligomerization of the chimera with EGF. This result suggested that the increase in lck activity observed following CD4 cross-linking is not due to a specific change in conformation of CD4 but rather to an aggregation of the CD4-associated lck. However, activation of lck by cross-linking CD4 independently of the TcR generates negative signals for activation of mature T cells (anergy) and occurs *in vivo* in a pathological situation (HIV infection) due to the interaction of gp120 with CD4. These observations suggest that the non-specific interaction of CD4 with MHC class II molecules in the periphery, does not induce an increase in lck tyrosine kinase activity which would have the potential to lead to anergy.

In the thymus, the CD4/lck complex was demonstrated to transmit intracellular signals. Indeed, the productive interaction between MHC class II and CD4 modulates TcR expression and the tyrosine phosphorylation of ζ (42,43). This result supports the hypothesis that the interaction of CD4 with MHC class II molecules can activate the CD4-associated lck and send intracellular signals. However, these studies do not exclude that a simultaneous interaction of the TcR and CD4 with the MHC molecules is taking place.

The question remains whether a cross-link of CD4 molecules occurs to activate lck when the TcR of mature T cells recognize their specific antigen. The observation that

MHC class II molecules can exist as dimers suggests that dimers of class II may cross-link CD4 and activate lck (11). Accordingly, it was proposed that interaction of CD4 with MHC class II molecules induces the dimerization of CD4 molecules (55). However, no direct demonstration of an increase in lck activity induced by such interaction of MHC class II and CD4 independently of TcR ligation in mature T cells has been reported. Determining whether lck is activated by transphosphorylation, like growth factor receptor tyrosine kinases, would support the requirement for CD4 cross-linking to activate lck during the antigen recognition process.

Of note, the increase in lck activity observed after either CD4 cross-linking or TcR stimulation is only in the order of 2-3 fold even though the presence of lck is absolutely required for initiation of T cell activation. This suggests that either a very localized activation of a few lck molecules is occurring following stimulation, and/or that lck may also regulate early events of T cell activation independently of its kinase activity. Interestingly, reconstitution of a CD4-dependent response of a T cell hybridoma to antigenic stimulation was achieved by a chimeric molecule consisting of the extracellular domain of CD4 and lck(65). Importantly, the response was also reconstituted by a chimera which was kinase dead but still possessed an intact SH2 domain. The SH2 domain of lck could thus generate signals through interactions with tyrosine phosphorylated proteins. These studies demonstrate that the CD4/lck complex can enhance T cell activation independently of the kinase activity of lck.

2) Several pieces of evidence suggest that another role for the association of lck with CD4 is to sequester lck, thereby preventing phosphorylation of its substrates in an unregulated fashion. Indeed, lck can generate signals regulating T cell activation independently of its association with CD4. In chapter 2, CD4 negative variants of the 2.10 T cell clone were found to be more sensitive to stimulation than CD4 positive variants. In these CD4 negative cells, lck is recruited to the TcR complex without help from the CD4/MHC class II interaction. How exactly this occurs is still obscure. A possible explanation would be that recognition of antigen by the TcR induces a conformational change of the CD3 and ζ chains that allows recruitment of lck and the subsequent tyrosine phosphorylation of these chains by lck. Alternatively, a higher basal phosphorylation of ζ by the free lck in resting CD4 negative T cells could reduce the threshold of stimulation required to activate CD4 negative T cells as compared to CD4 positive T cells.

lck can also act independently of its association with CD4 in the thymus. Indeed, overexpression of lck in the thymus was demonstrated to inhibit the rearrangement of the TcRβ locus and to induce lymphomas (1). This effect of lck was still observed when a

mutant of lck which cannot associate with CD4 was overexpressed, demonstrating that the signal was not generated through CD4 (33). Moreover, positive selection of thymocytes can occur when a mutant CD4 which is not associated with lck is expressed instead of wild type CD4 (28).

Taken together, these studies demonstrate that lck can act without being activated through CD4 cross-linking. In fact, a role of its association with CD4 would be to sequester lck as demonstrated in chapter 2 and 3. This sequestration could be important in preventing immune dysfunction. Indeed, one can envision that in the periphery, a lack of sequestration of lck by CD4 could render T cells more easily activable and/or prime them by inducing a basal phosphorylation of ζ. This could bypass the requirement for other co-stimulatory signals to activate T cells and lead to autoimmunity. Moreover, lck can generate mitogenic signals and lymphoproliferative diseases could result from deregulation of its activity.

In accordance with the sequestration model, a recent study suggests that most of the lck is not free inside the cell (44). The authors showed that overexpression of CD4 in thymocytes does not result in an increased number of lck molecules associated with CD4. A diminution in the ratio of lck to CD4 molecules is rather observed. This suggests that all lck molecules are associated with other molecules and that the amount of free lck is tightly regulated *in vivo*.

The association of CD4 with lck may also be required to ascertain that lck is in the close proximity of the TcR signaling complex upon antigenic recognition. In chapter 2, the response of CD4 positive clones to anti-TcR Abs could only be observed if CD4 was co-aggregated with the TcR. These results suggest that the CD4/lck complex provides an obligatory signal for the coupling of the TcR with the CD3 signaling complex and must be in close contact with the TcR for the initiation of T cell activation. In antigenic stimulation, the MHC class II molecule which is both recognized by the TcR and CD4 would allow these TcR/ CD4-lck interactions to take place. Importantly, these results support the model that CD4 and the TcR recognize the same MHC class II molecules on the APC (co-receptor function of CD4). In addition, these observations were made with both murine and human CD4 molecules demonstrating the conservation across species of this role of CD4.

In conclusion, the association of lck with CD4 would regulate the kinase activity by sequestering lck molecules and preventing unregulated phosphorylations of its substrates. In addition, this interaction would secure the co-aggregation of lck with the TcR signaling complex to initiate T cell activation. Further work is required however to determine if the interaction between MHC class II molecules and CD4 molecules either

simultaneously or independently of the recognition of antigen by the TcR , activates lck tyrosine kinase activity.

2. What is the extracellular regulatory function of CD4?

In chapter 2, we showed that CD4 is sequestering lck from the TcR and that a co-aggregation between CD4/lck and the TcR is required to initiate T cell activation. To determine the role of the extracellular domain of CD4 apart from interacting with MHC class II molecules (chapter 3), we designed a chimera consisting of the extracellular domain of the EGFR and the transmembrane and cytoplasmic domains of CD4. This chimera was demonstrated to associate with the same levels of lck as wild type CD4. However, the chimera was not downregulating anti-TcR induced proliferation as opposed to wild type CD4. This result suggested that the EGFRCD4 chimera lacked a regulatory function that CD4 possesses which maps to its extracellular domain.

Results presented in chapter 4 demonstrated that gp120 inhibits the stimulation by antigen of wild-type CD4 and double cysteine (which lacks the association with lck) expressing clones. Moreover, the stimulation of clones expressing the EGFRCD4 chimera was not inhibited by either EGF which stimulates lck tyrosine kinase activity or anti-EGFR Ab. Thus, the inhibition observed with gp120 was not due to an activation of lck or a sequestration of lck from the TcR by the cytoplasmic domain of CD4. This inhibition requires again the extracellular domain of CD4. Since the response of this T cell clone is not increased by the presence of an MHC class II-CD4 interaction, this inhibition cannot either be explained by a decrease in adhesion between the T cell and the APC.

Taken together, our results suggest that the extracellular domain of CD4 interacts with another T cell surface molecule which is involved in the generation of early activation events. This is also supported by studies proposing that CD4 regulates activation events independently of its association with lck. First, reconstitution of a CD4-dependent response to antigenic stimulation of a T cell hybridoma was achieved by expressing a mutant CD4 which is not associated with lck (67). Moreover, anti-CD4 inhibition of antigenic stimulation of these hybridomas was also occurring in cells expressing this mutant of CD4. In the thymus, CD4 expression is required to observe a normal level of CD4 positive T cells in the periphery. Interestingly, overexpression of a CD4 which does not associate with lck in CD4 knock-out mice allows the positive selection of these cells, again suggesting that CD4 can act independently of its association with lck(28).

The first protein candidate for this regulatory activity associated with CD4 that comes to mind is the TcR complex. Indeed, association with the TcR was previously demonstrated by co-capping, co-modulation and co-precipitation experiments (12,15,29,30,54). However, several pieces of evidence suggest that the interaction between the TcR and CD4 occurs intracellularly. Indeed, the resonance transfer experiments demonstrated that lck association with CD4 was required to observe an interaction between CD4 and the TcR complex (15). Recent biochemical data shows that the SH2 domain of the CD4-associated lck interacts with ζ-associated tyrosine phosphorylated ZAP (19,61). The association of ZAP with lck can be correlated with co-capping of the TcR, CD4 and lck in these experiments. The intracellular cross-talk between the TcR and the CD4-associated lck is not surprising since it was previously demonstrated that the TcR sends intracellular signals to CD8, which is also associated with lck, and modulates its binding to MHC class I molecules (49). The interaction between the TcR complex and CD4 would then be an indirect one involving intermediate molecules such as ZAP and lck.

Confirmation of an intracellular interaction between CD4 and the TcR complex would come by the use of the mutant CD4 molecules described in this thesis. Indeed, an interaction between the TcR and the EGFRCD4 chimera, which is associated with lck, should be observed following TcR stimulation. Alternatively, the mutant of CD4 which does not associate with lck, the double cysteine mutant, would not be expected to interact with the TcR complex. However, this does not rule out that an intracellular interaction between lck and ZAP may be stabilized by an interaction between the extracellular domains of CD4 and the TcR complex.

The result demonstrating that clones expressing the EGFRCD4 chimera can be stimulated to proliferate by anti-TcR Abs as opposed to wild type CD4 expressing clones , support the model in which the regulatory role of the extracellular domain of CD4 is a sequestration of a molecule away from the TcR and not a lack of association of this molecule with the TcR. The results presented in this thesis thus favor the hypothesis that the extracellular domain of CD4 associates with a T cell surface molecule other than the TcR.

Another interesting candidate for this regulatory molecule is CD45. The tyrosine phosphatase CD45 was found to be associated with a number of T cell surface molecules, including CD4 (18,41). The nature of these interactions is not defined but the interaction between CD45 and CD4 seems to be isoform specific. Isoforms of CD45 are defined by the differential use of exons coding for its extracellular domain. It is thus possible that CD4 and CD45 associate with each other`s extracellular domains and that this corresponds

to the regulatory function of the extracellular domain of CD4 that we have identified in the above described experiments. Moreover, several studies have shown that anti-CD45 Abs can inhibit activation of T cells (22,32). The inhibition of antigenic stimulation by gp120 observed in our experiments could thus result from a similar perturbation of the CD45 activity associated with CD4.

Interestingly, our results suggest that presence of both the extracellular domain of CD4 and the association with lck is required to observe down regulation of anti-TcR induced proliferation. Indeed, in the case of the EGFRCD4 chimera, only lck is sequestered and anti-TcR stimulation induces proliferation of T cells. Similarly, the double cysteine mutant has the extracellular domain of CD4 but lacks lck association, and allows anti-TcR induced proliferation. In contrast, wild type CD4 sequesters both lck and another molecule through its external domain, and inhibits anti-TcR induced growth. These results suggest that there is a compensation between lck sequestration and the regulatory function of the extracellular domain of CD4. Interestingly, a similar compensation between CD45 and lck has been reported in CD45 negative cell lines (17). These cell lines cannot be stimulated to produce IL-2 using anti-TcR Abs. However, co-crosslinking of the CD4/lck complex with the TcR rescues stimulation by anti-TcR Abs.

According to our model, CD4 would thus regulate the amount of CD45 phosphatase activity and the amount of lck that are in the proximity of the TcR signaling complex. This would ensure an optimal amount of tyrosine kinase and phosphatase enzymatic activity required for the initiation of T cell activation is present. Confirmation of this hypothesis should be done by differential co-precipitations of CD45 with CD4 and the EGFRCD4 chimera. This model is supported by functional data showing that reconstitution of an antigen specific response of a CD45 negative T cell clone by low mwt isoforms of CD45 results in a substantially greater response to antigenic stimulation than that elicited in clones transfected with high mwt isoforms of CD45 (48). Interestingly, only the low mwt isoforms of CD45 were found to associate with CD4 in these clones, suggesting that the association of CD45 with CD4 potentiates T cell responses.

SIGNALS GENERATED THROUGH CD4 REGULATE HIV REPLICATION

1. Modulation of HIV replication by CD4: what is the mechanism and who does it benefit?

HIV infection is also regulated by signals originating from CD4. The work presented in chapter 5 demonstrates that the association of lck with CD4 directly modulates HIV replication. Indeed, HIV was replicating more efficiently in cells bearing a CD4 which does not associate with lck. The results suggest that the decreased viral replication in wild type CD4 expressing cells is not due to sequestration of lck by CD4 (lack of a positive signal) but rather to an increase in tyrosine kinase activity of the CD4-associated lck induced by gp120 (negative signal). The signal generated through lck then leads to a down regulation of transcription from the LTR of HIV. This model is supported by a report demonstrating that treatment of infected cells with anti-CD4 Abs not directed to the gp120 binding site, led to inhibition of transcription from the HIV LTR and down regulation of viral replication (9).

This modulation of transcription from the HIV LTR is reminiscent of the effect of gp120 on T cell activation (figure 1). Indeed work presented in chapter 4 and other reports have demonstrated that gp120 could inhibit stimulation of T cells by both antigen and anti-TcR Ab. In addition, inhibition of anti-TcR stimulation of resting PBLs by gp120 is accompanied by a lack of IL-2 production(50). This is not surprising since some of the transcription factors involved in directing transcription from the IL-2 promoter and the HIV LTR are the same. So the modulation of HIV replication by gp120 occurs at two levels: direct inhibition of transcription from the HIV LTR and indirect inhibition by preventing activation of T cells (figure 2). However, caution must be taken in that although both promoters use the same transcription factors, fine regulation of transcription for these two promoters is probably different. Indeed, we have shown that inhibition of transcription from the HIV LTR requires lck association with CD4, while the inhibition of antigenic stimulation can occur in the absence of lck association with CD4. This suggests that different signals can regulate the two promoters.

The involvement of lck tyrosine kinase activity was further confirmed in recent experiments showing that HIV replication is higher in CD45 negative variants of the T cell line Jurkat as compared to CD45 positive Jurkat cells (S. Gratton, unpublished results). This result makes a correlation between the lack of activity of lck in CD45 negative cell lines and higher replication of HIV. More direct evidence came from the infection of cells which co-express a CD4 molecule lacking its cytoplasmic domain (thus association with

lck) and the EGFRCD4 chimera (S. Gratton, unpublished results). As described above, EGF stimulates the kinase activity of the chimera-associated lck. Interestingly, viral replication in these cell lines was completely abrogated when cells were grown in media supplemented with Fetal Calf Serum (FCS) which contains EGF. In addition, lck was found to be hyperphosphorylated on tyrosine residues in these cell lines in the presence of FCS. These results are consistent with an increase in lck tyrosine phosphorylation induced by external signals leading to an inhibition of HIV replication.

The gp120 treatment of CD4 positive T cells was demonstrated to inhibit DNA binding activities of several transcription factors, including NFκB, NF-AT and AP-1 normally induced following stimulation through the TcR (26). Moreover, higher levels of NF-κB are found in the nucleus of CD45 negative Jurkat cells in comparison with that found in CD45 positive Jurkat cells (7). The observed inhibition of transcription from the HIV LTR thus could be due to perturbation of the activity of these transcription factors. Experiments studying directly the effect of CD4 cross-linking on the HIV LTR can be done. The use of viral LTR or proviral clones with specific deletions of transcription factor binding sites will be useful tools to identify which transcription factors are regulated by lck.

Alternatively, gp120 ligation of CD4 could induce the release of cytokines which would modulate HIV replication (figure 2). This is supported by the recent report demonstrating the induction of cytokine expression by cross-linking of CD4 with gp120 (51). Interestingly, some of these cytokines (interferon-γ and tumor necrosis factor-α) can induce the expression of the Fas antigen in T cells . The Fas antigen is a cell surface molecule that induces apoptosis of the cell it is expressed on upon recognition of its ligand. Thus, another potential mechanism of down regulation of HIV replication by CD4 would be by inducing apoptosis of CD4 positive cells. However, these cytokines were also demonstrated to upregulate HIV replication on their own, suggesting that regulation of HIV replication through this pathway may be more complex than first believed.

These observations raise one interesting question: is the down regulation of HIV replication by the CD4 beneficial for the host or the virus (see figure 2)? This modulation of HIV replication would be beneficial for the host in that a decrease in the size of viral progeny would lead to a decreased number of newly infected cells in the next round of replication. The induction of apoptosis of CD4 T cells by gp120 would also result in a decrease in the total number of infectable T cells. On the other hand, this reduction in CD4 T cells would also be accompanied by a decrease in host immune response against HIV and other opportunistic diseases. Thus, the signal generated by gp120 would prevent

a rapid clearance of infected cells by the host immune system, but it would also allow the rapid deterioration of the host due to opportunistic diseases.

HIV infection is characterized by a long clinically asymptomatic period. This favors the pathogen as the host can transmit the virus during that period without having the knowledge of being infected. However, it was demonstrated that during that period, HIV replication is going on in the secondary lymphoid organs challenging the latency concept that was first put forward (20,52). This observation does not eliminate the possibility that latently infected cells are found in HIV infected patients. More work is needed to prove or disprove their existence *in vivo*. Latency would allow the virus to escape the host immune system and keep a reservoir of infection. If no viral proteins are produced, the infected cells cannot be recognized as foreign and be killed by the immune system. This ensures that the viral infection cannot be cleared away completely. The negative signal could also diminish the viral production generated per infected cells. This would allow the host to remain in the clinically asymptomatic phase longer and allow a larger propagation of the virus.

Although we have experimented on two viral clones, study of clinical isolates would be interesting in assessing whether this regulation by CD4/lck is a conserved feature of HIV. The rate of mutagenesis of HIV *in vivo* and the rapid appearance of variants which are resistant to anti-viral drugs following treatments (25,63), suggest that if conserved in clinical isolates, this modulation of replication by CD4 may be beneficial for the virus.

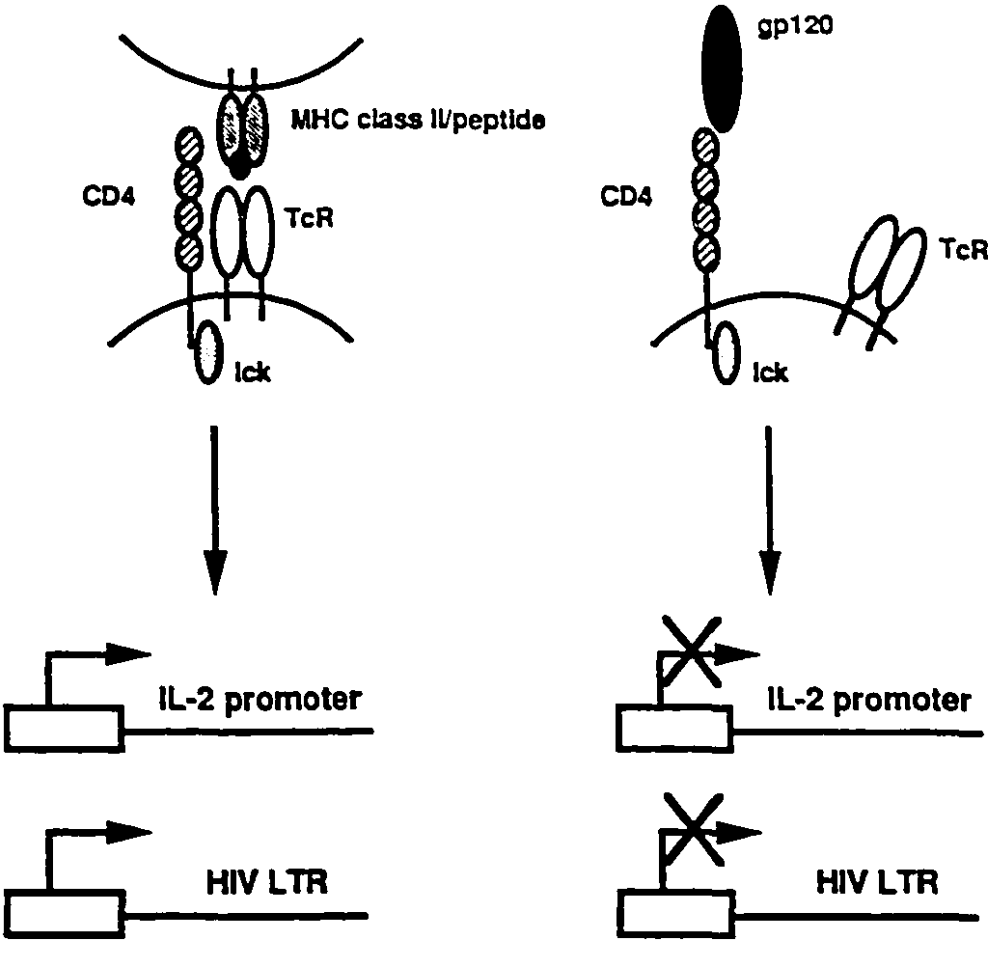


Figure 1

The Il-2 promoter and the HIV LTR are regulated by similar signals. Activation of lck in the context of antigenic stimulation will induce their expression. In contrast, activation of lck independently of ligation of the TcR will result in inhibition of transcription from both promoters.

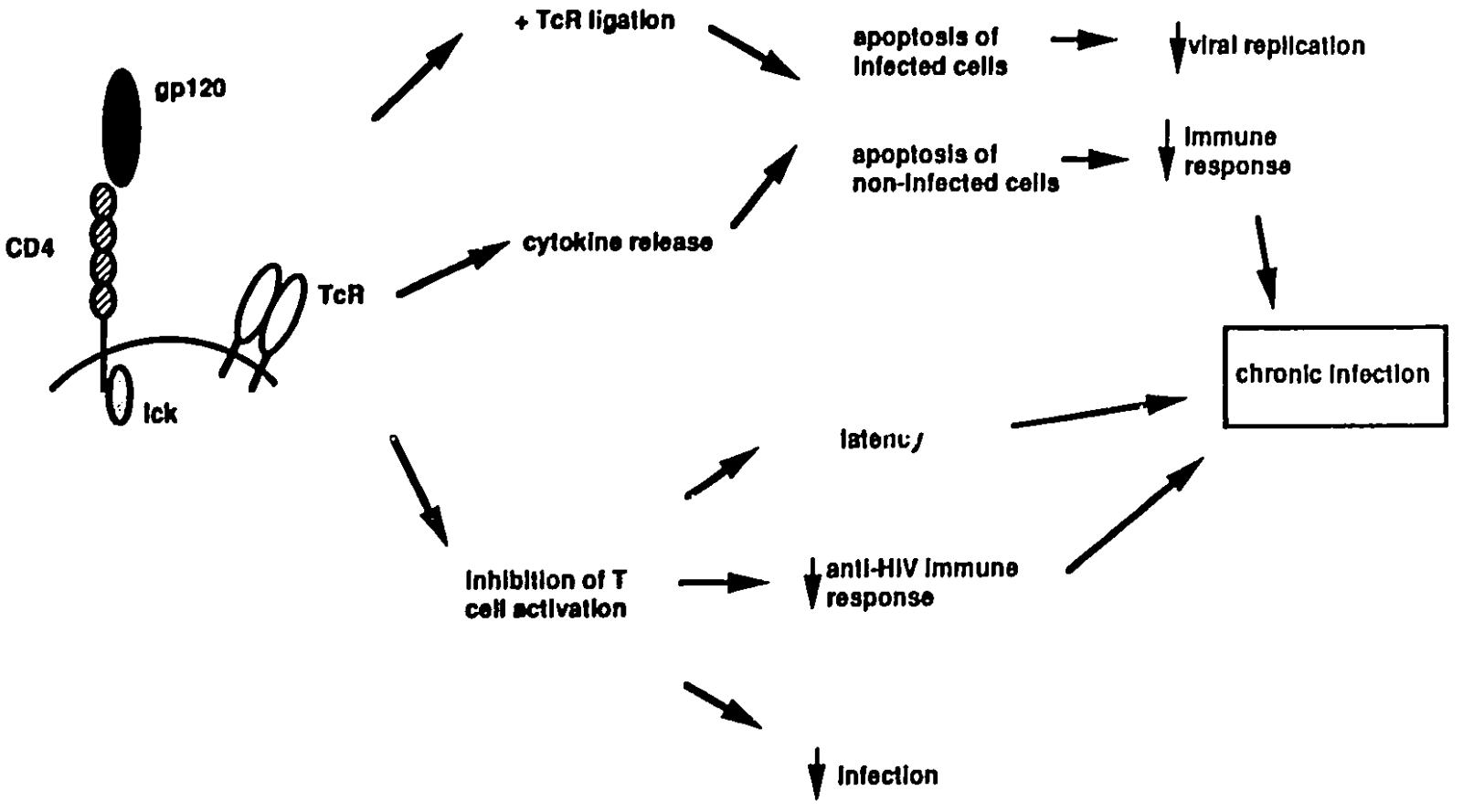


Figure 2

Possible systemic effects of gp120.

2. Mechanism of the positive effect of Nef on HIV: is the down regulation of expression of CD4 involved?

The HIV *nef* gene is expressed early in the viral life cycle. Early studies first described Nef as a negative regulator of transcription driven from the HIV LTR, and implicated this regulatory protein in the phenomenon of latency (2,14,35,47). However, several studies contradicted these by reporting that Nef was having either no effect on the HIV LTR or was increasing HIV replication by augmenting the infectivity of the progeny virus (13,16,24,40,60,66). These contradictory reports may be explained by new data which suggest that the effect of Nef on HIV replication depends on the activation state of the cell at the time of the infection. Indeed, the positive effect of Nef is more easily observed *in vitro* when resting PBLs are infected in contrast with infection of mitogen activated PBLs or cell lines (40,60).

The report showing that Nef is associated with a serine kinase further supports the hypothesis that the effect of Nef is elicited by the modification of intracellular signaling pathways (58). More recently, it was reported that proline rich domains of Nef could interact with SH3 containing proteins, including the tyrosine kinase hck (56). Importantly, mutations that abrogate the binding of Nef to SH3 domains also abrogate the positive effect of Nef on HIV replication *in vitro*. Moreover, Nef was found to directly affect T cell activation events. Again, the reports are contradictory. In cell lines stably expressing Nef, the DNA binding activity of AP-1 and NF-κB is inhibited, resulting in decreased transcription from the IL-2 promoter (37,45,46). In transgenic mice expressing Nef in thymocytes, an enhancement or an inhibition of anti-TcR stimulation was observed (10,34,59). The differences in phenotypes may be explained by the use of different promoters directing transcription of the Nef transgene at different stages of maturation of thymocytes. Thus again the activation state of the T cell would direct the outcome of the effect of Nef.

Another interesting model was suggested by Bauer et al (6). Their data in T cell lines show that the effect of Nef on T cell activation depends on the subcellular localization of Nef. When Nef is mainly intracellular, inhibition of T cell activation is observed. In contrast, cells expressing mainly a membrane bound form of Nef are more susceptible to TcR stimulation and are induced to express activation markers in a constitutive way. This expression of activation markers induced by Nef was also observed in peripheral T cells in a Nef transgenic mouse line (34). Moreover, these cells are not long lived and die, or mutate their Nef gene to survive indicating that the presence of Nef at the cell membrane is deleterious for cells. However, the relevance of this model to infection of resting PBLs is

not yet determined. Taken together, these evidences suggest that Nef exerts a positive effect on HIV replication by modulating activation pathways of T cells. By lowering the activation threshold of T cells, Nef would thus increase the susceptibility of a resting T cell to be productively infected by HIV (figure 3).

Alternatively, Nef could act by preventing apoptosis of infected cells (figure 3). Indeed, apoptosis is observed in both infected and non-infected PBLs isolated from HIV patients (3,23,31,39). Exposure to gp120 or tat was demonstrated to lead to apoptosis of T cells *in vitro* (64). Nef could, by associating with signaling molecules, inhibit directly apoptosis pathways. This would allow infected cells to produce a large amount of virus before cell death. In addition, Nef could prevent the apoptosis of latently infected cells and thus keep a viral reservoir. There is some precedent for anti-apoptotic genes encoded by viruses. Indeed, the cow pox virus *crmA* inhibits apoptosis induced by several experimental conditions (53).

Of course the *in vitro* models merely reflect *in vivo* infection. Either pre-activated or resting T cells that encounter their specific antigen shortly after viral entry, can be infected *in vivo*. As Nef cannot be detected in the virions by conventional methods, proviral DNA must be integrated to get expression of Nef. An effect on the coupling of TcR with the IL-2 promoter is thus irrelevant because Nef appears after this activation step has taken place. However, by promoting IL-2 production Nef could prevent premature apoptosis of infected T cells due to a lack of growth factor.

Infection of macaques by SIV was demonstrated to require the presence of a functional *nef* gene for production of high viral loads and establishment of pathogenesis (27). A comparative analysis of T cell phenotypes between animals infected with a Nef positive or a Nef negative virus regarding the induction of markers and susceptibility to antigenic stimulation or cytokine stimulation, could help us correlate these two phenomena. Moreover, neonates seem not to require the presence of Nef for establishment of infection (5). Interestingly, neonates have a more activated immune system than adults. If Nef acts by lowering the activation threshold of T cells, infection of neonates T cells would thus not require this enhancing effect of Nef that adults require. Investigation on what is different in the immune system of neonate and adult monkeys should also help in understanding the effect of Nef.

We have demonstrated that the association of lck with CD4 down regulates HIV replication (chapter 5). Nef was found to down regulate cell surface expression of CD4. Recently, it was reported that mutagenesis of the proline rich region of Nef resulted in a Nef which no longer enhances in vitro replication of HIV (56). Interestingly, this mutant Nef still could induce down regulation of cell surface expression of CD4. However, we

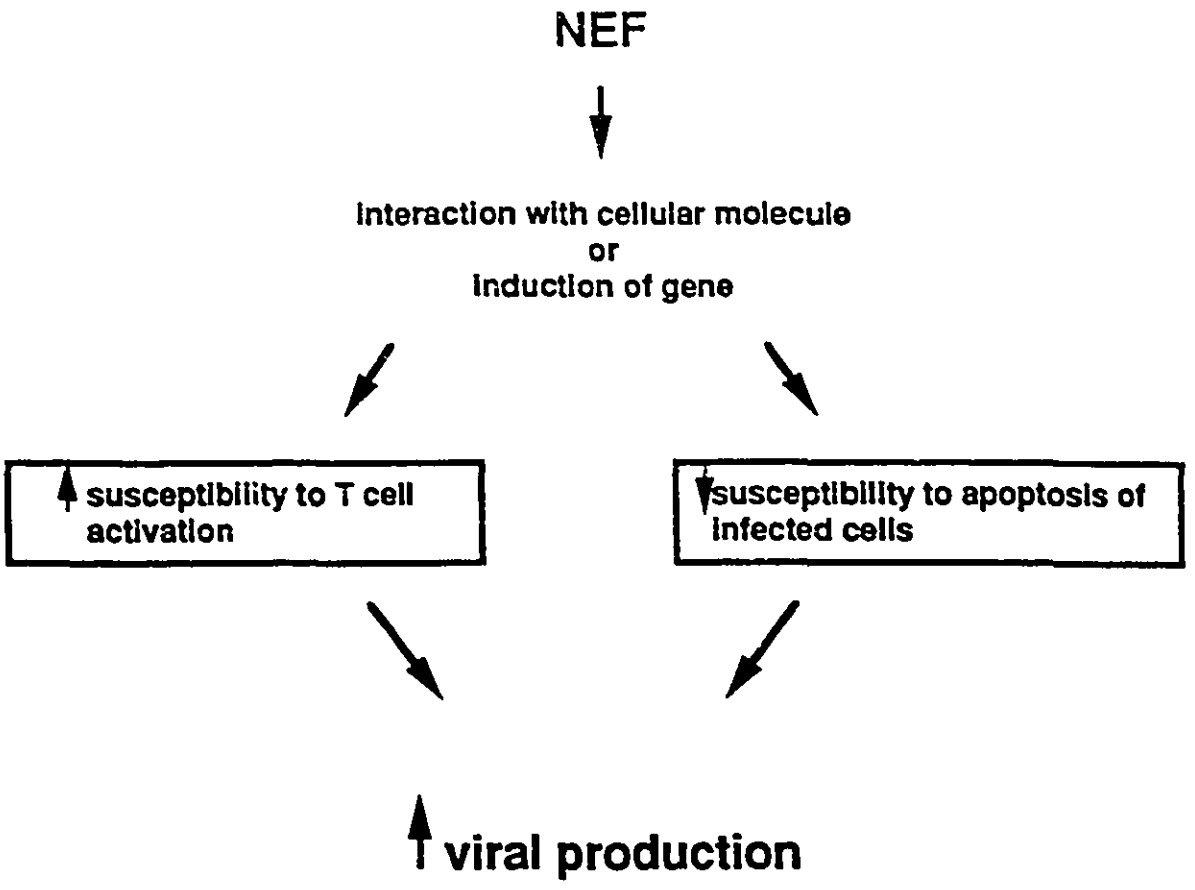


Figure 3

Possible involvement of modulation of signaling pathways in the positive effect of Nef on HIV replication.

feel that this does not exclude the possibility that down regulation of the CD4/lck complex from the cell surface also contributes to the positive effect of Nef. Indeed, down regulation of expression of CD4 is a conserved property of Nef encoded by several clinical isolates, suggesting that it is an important feature for HIV (4,21,38). Moreover, the decrease in cell surface expression of CD4 induced by Nef would abolish the negative signal generated through the CD4/lck association (figure 4). Testing of this hypothesis is under way in several laboratories. CD4 negative cells are being transfected with either wild type CD4 or mutants of CD4 that have disrupted association with lck and/or lost the susceptibility to down regulation induced by Nef. These cells will be infected with two isogenic HIV clones one of which lacks the coding sequence of Nef. By comparing the levels of viral production between each mutants, we will assess the contribution of lck association with CD4 and down regulation of expression of CD4 to the positive effect of Nef on HIV replication.

Interestingly, the dissociation of lck from CD4 in HIV infected cells is not due to Nef (chapter 7). Nef rather seems to induce the internalization of the whole CD4/lck complex. However lck is not degraded in the presence of Nef. This results in more lck free of CD4 in cells infected with a Nef positive virus as compared to cells infected with a virus lacking coding sequences for Nef . As we have already discussed above, lck is regulated through its association with CD4. Cells in which lck is not associated with CD4 are more sensitive to stimulation by anti-TcR Abs. Thus, an increase in activation of T cells by inhibiting the sequestration of lck by CD4 could also explain the increase in viral replication in the presence of Nef (figure 4).

Finally, it was recently proposed that abnormal targeting of CD4 to the lysosomes allows presentation of cryptic CD4 peptide by MHC class II molecules in T cells (57). This could stimulate autoreactive T cells specific for CD4 which are normally silent in the periphery. Degradation of CD4 induced by Nef could thus also lead to autoimmunity against CD4 T cells.

We have demonstrated that Nef also down regulates the expression of CD4 by inhibiting CD4 expression at the level of synthesis (chapter 6). This inhibition occurs early in the biosynthetic pathway as treatment with brefeldin A did not abrogate the effect of Nef. Interestingly, an interaction between Nef and βCOP which is implicated in transport of newly synthesized proteins has been previously observed (8). This suggests that the inhibition of synthesis may be wide spread and target a number of molecules. This may be a mechanism by which HIV would favor synthesis and maturation of viral proteins over cellular proteins. This inhibition of synthesis of CD4 would also prevent its association

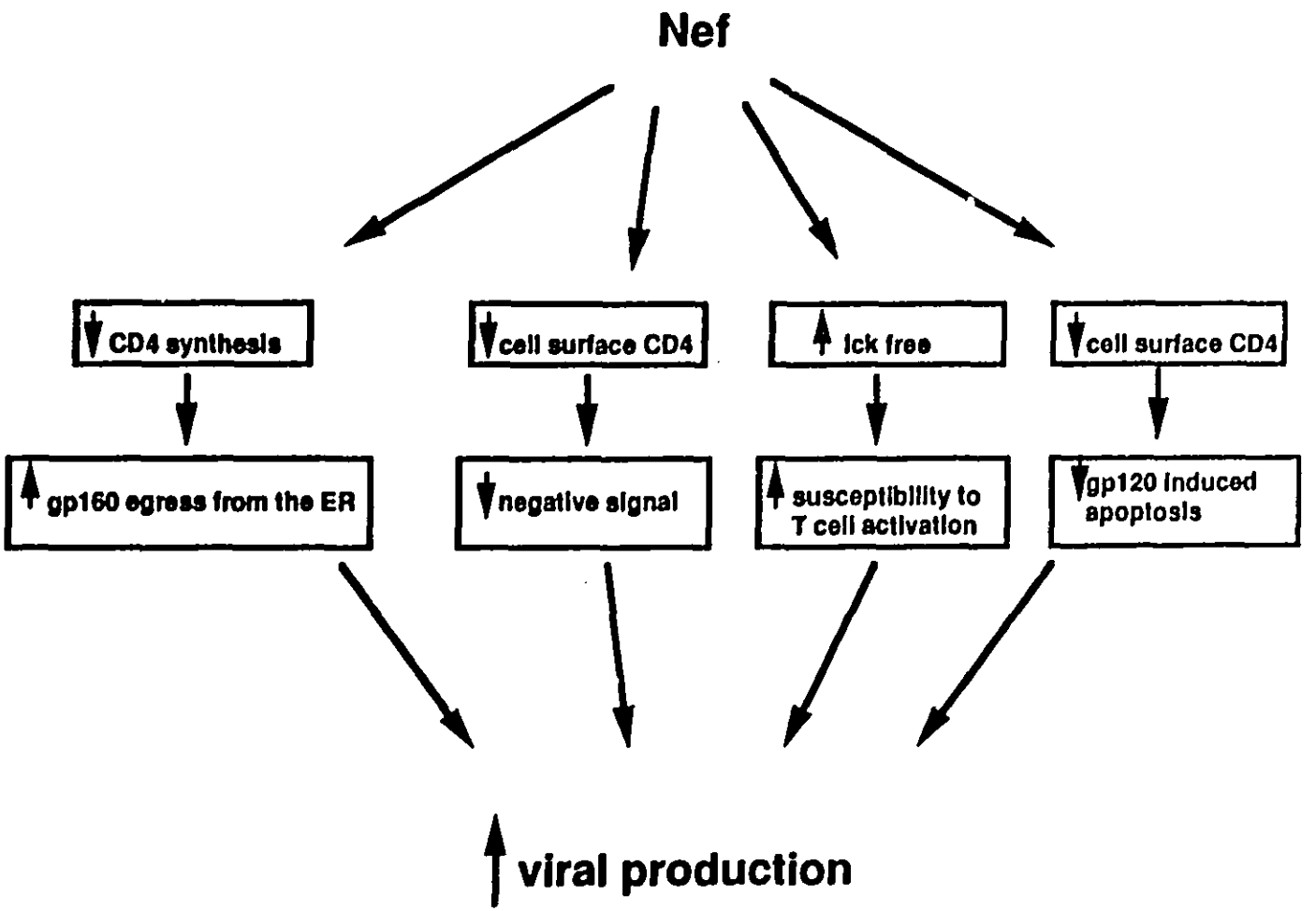


Figure 4
Possible implication of down regulation of expression of CD4 in the positive effect of Nef on HIV replication

with gp160, thus increasing the egress of gp160 from the ER. This could also explain the increase in infectivity of virions produced in the presence of Nef (figure 4).

Taken together, there is strong evidence that down regulation of expression of CD4 induced by Nef is implicated in the effect of Nef on HIV replication. The fact that Nef expression is required for the establishment of disease in adult monkeys suggest that Nef negative viruses are potential vaccines. It is thus imperative to fully characterize the effect of Nef on T cell activation pathways.

CONCLUDING REMARKS

The HIV has evolved to respond to cellular activation signals and to modulate these signals for its own purpose. By choosing CD4 as its receptor, it has targeted a molecule which regulates the activation of a crucial player of the immune system. This certainly has given the advantage to the virus of controlling its level of replication. The length of the clinical latency period suggests that HIV favors a slow progressive infection of the CD4 T cell pool rather than an aggressive rapid infection. Several HIV proteins, such as gp120 and Nef, interact with CD4, generating signals and modulating its normal function. This allows the virus to control the immune response against itself. In addition, these signals modulate HIV replication either by acting directly on transcription from its LTR or indirectly, by controlling the activation of T cells which is required to get infection. Further work is required however, to understand which regulatory pathways are benefic for the host and which are for the virus, such that therapies directly targeted at modulating these pathways may be designed.

REFERENCES

1. Abraham, K. M., S. D. Levin, J. D. Marth, K. A. Forbush, and R. M. Perlmutter. 1991. Thymic tumorigenesis induced by overexpression of $p56^{lck}$. Proc. Natl. Acad. Sci. USA. 88:3977-3981.

2. Ahmad, N. and S. Venkatesan. 1988. Nef protein of HIV-1 is a transcriptional repressor of HIV-1 LTR. Science 241:1481-1485.

3. Ameisen, J. C. and A. Capron. 1991. Cell dysfunction and depletion in AIDS: the programmed cell death hypothesis. Immunology Today 12:102-105.

4. Anderson, S., D. C. Shugars, R. Swanstrom, and J. V. Garcia. 1993. Nef from primary isolates of human immunodeficiency virus type 1 suppresses surface CD4 expression in human and mouse T cells. J. Virol. 67:4923-4931.

5. Baba, T. W., Y. S. Jeong, D. Pennick, R. Bronson, M. F. Green, and R. M. Ruprecht. 1995. Pathogenicity of Live, attenuated SIV after mucosal infection of neonatal macaques. Science 267:1820-1825.

6. Bauer, A. S., E. T. Sawai, P. Dazin, W. J. Fantl, C. Cheng-Mayer, and B. M. Peterlin. 1994. HIV-1 Nef leads to inhibition or activation of T cells depending on its intracellular localization. Immunity 1:373-384.

7. Baur, A., S. Garber, and B. M. Peterlin. 1994. Effects of CD45 on NF-κB: implications for replication of HIV-1. J. Immunol. 152:976-983.

8. Benichour, S., M. Bomsel, M. Bodeus, H. Durand, M. Doute, F. Letourneur, J. Camonis, and R. Benarous. 1994. Physical interaction of the HIV-1 Nef protein with β-COP, a component of non-clathrin-coated vesicles essential for membrane traffic. J. Biol. Chem. 269:1-4.

9. Benkirane, M., P. Corbeau, V. Housset, and C. Devaux. 1993. An antibody that binds the immunoglobulin CDR3-like region of the CD4 molecule inhibits provirus transcription in HIV-infected T cells. EMBO J. 12:4909-4921.

10. Brady, H. J., D. J. Pennington, C. G. Miles, and E. A. Dzierzak. 1993. CD4 cell surface downregulation in HIV-1 Nef transgenic mice is a consequence of intracellular sequestration. EMBO J. 12:4923-4932.

11. Brown, J. H., T. S. Jardetzky, J. C. Gorga, L. J. Stern, R. G. Urban, J. L. Strominger, and D. C. Wiley. 1993. Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. Nature 364:33-39.

12. Burgess, E. K., A. D. Odysseos, C. Zalvan, B. J. Drucker, P. Anderson, S. E. Schlossman, and C. E. Rudd. 1991. Biochemical identification of a direct physical interaction between the CD4:p56^{lck} and Ti(TcR)/CD3 complexes. Eur. J. Immunol. 21:1663-1668.

13. Chang, J. C., L. Zhang, T. L. Edgerton, and A. M. Kaplan. 1990. Heterogeneity in direct cytotoxic function of L3T4 T cells. TH1 clones express higher cytotoxic activity to antigen-presenting cells than TH2 clones. Journal of Immunology 145:409-416.

14. Cheng-Mayer, C., P. Iannello, K. Shaw, P. A. Luciw, and J. A. Levy. 1989. Differential effects of *nef* on HIV replication: implications for viral pathogenesis in the host. Science 246:1629-1632.

15. Collins, T. L., S. Uniyal, J. Shin, J. L. Strominger, R. S. Mittler, and S. J. Burakoff. 1992. p56^{lck} association with CD4 is required for the interaction between CD4 and the TcR/CD3 complex and for optimal antigen stimulation. J. Immunol 148:2159-2162.

16. de Ronde, A., B. Klaver, W. Keulen, L. Smit, and J. Goudsmit. 1992. Natural HIV-1 Nef accelerates virus replication in primary human lymphocytes. Virology 188:391-395.

17. Deans, J. P., S. B. Kanner, R. M. Torres, and J. A. Ledbetter. 1992. Interaction of CD4:lck with the T cell receptor/CD3 complex induces early signaling events in the absence of CD45 tyrosine phosphatase. Eur. J. Immunol. 22:661-668.

18. Dianzani, U., U. Reddoglia, F. Malavasi, M. Bradardo, A. Pileri, C. A. Janeway, and K. Bottomly. 1992. Isoform-specific associations of CD45 with accessory molecules in human T lymphocytes. Eur. J. Immunol. 22:365-371.

19. Duplay, P., M. Thome, F. Herve, and O. Acuto. 1994. $p56^{lck}$ interacts via its src homology 2 domain with the ZAP-70 kinase. Journal of Experimental Medicine 179:1163-1172.

20. Embretson, J., M. Zupanic, J. L. Ribas, A. Burke, P. Racz, K. Tenner-Racz, and A. T. Haase. 1993. Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. Nature 362:359-361.

21. Garcia, J. V., J. Alfano, and A. D. Miller. 1993. The negative effect of human immunodeficiency virus type 1 Nef on cell surface CD4 expression is not species specific and requires the cytoplasmic domain of CD4. J. Virol. 67:1511-1516.

22. Goldman, S. J., S. Uniyal, L. M. Ferguson, D. E. Golan, S. J. Burakoff, and P. A. Kiener. 1992. Differential activation of phosphotyrosine protein phosphatase activity in a murine T cell hybridoma by monoclonal antibodies to CD45. Journal of Biological Chemistry. 267:6197-6204.

23. Groux, H., G. Torpier, D. Montle, Y. Mouton, A. Capron, and J. C. Ameisen. 1992. Activation-induced death by apoptosis in CD4+ T cells from human immunodeficiency virus-infected asymptomatic individuals. Journal of Experimental Medicine 175:331-40.

24. Hammes, S. R., E. P. Dixon, M. H. Malim, B. R. Cullen, and W. C. Greene. 1989. Nef protein of human immunodeficiency virus type 1: evidence against its role as a transcriptional inhibitor. Proc. Natl. Acad. Sci. USA 86:9549-9553.

25. Ho, D. D., A. U. Neumann, A. S. Perelson, W. Chen, J. M. Leonard, and M. Markowitz. 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature 373:123-126.

26. Jabado, N., F. LeDeist, A. Fisher, and C. Hivroz. 1994. Interaction of HIV gp120 and anti-CD4 antibodies with the CD4 molecule on Human CD4+ T cells inhibits the binding activity of NF-AT,NF-κB,and AP-1, three nuclear factors regulating IL-2 gene enhancer activity. Eur. J. Immunol. 24:2646-2652.

27. Kestler, H. W., 3d, D. J. Ringler, K. Mori, D. L. Panicali, P. K. Sehgal, M. D. Daniel, and R. C. Desrosiers. 1991. Importance of the *nef* gene for maintenance of high virus loads and for development of AIDS. Cell 65:651-662.

28. Killeen, N. and D. R. Littman. 1993. Helper T-cell development in the absence of CD4-p56^{lck} association. Nature 364:729-732.

29. Kupfer, A., S. J. Singer, C. J. Janeway, and S. L. Swain. 1987. Coclustering of CD4 (L3T4) molecule with the T-cell receptor is induced by specific direct interaction of helper T cells and antigen-presenting cells. Proc. Natl. Acad. Sci. USA. 84:5888-5892.

30. Kupfer, A., S. L. Swain, C. A. Janeway, and S. Singer,J. 1986. The specific direct interaction of helper T cells and antigen presenting B cells. Proc. Natl. Acad. Sci. USA 83:6080-6083.

31. Laurent-Crawford, A. G., B. Krust, S. Muller, Y. Riviere, M. A. Rey-Cuille, J. M. Bechet, L. Montagnier, and A. G. Hovanessian. 1991. The cytopathic effect of HIV is associated with apoptosis. Virology 185:829-39.

32. Ledbetter, J. A., N. K. Tonks, E. H. Fisher, and E. A. Clark. 1988. CD45 regulates signal transduction and lymphocyte activation by specific association with receptor molecules on T or B cells. Proc. Natl. Acad. Sci. U. S. A. 85:8628-8632.

33. Levin, S. D., K. M. Abraham, S. J. Anderson, K. A. Forbush, and R. M. Perlmutter. 1993. The protein tyrosine kinase p56^{lck} regulates thymocyte development independently of its interaction with CD4 and CD8 coreceptors. J. Exp. Med. 178:245-255.

34. Lindemann, D., R. Wilhelm, P. Renard, A. Althage, R. Zinkernagel, and J. Mous. 1994. Severe immunodeficiency associated with a human immunodeficiency virus 1 NEF/3'-long terminal repeat transgene. J. Exp. Med. 179:797-807.

35. Luciw, P. A., C. Cheng-Mayer, and J. A. Levy. 1987. Mutational analysis of the human immunodeficiency virus: the *orf*-B region down regulates virus replication. Proc. Natl. Acad. Sci. USA 84:1434-1438.

36. Luo, K. and B. M. Sefton. 1990. Cross-linking of T-cell surface molecules CD4 and CD8 stimulates phosphorylation of the lck tyrosine protein kinase at the autophosphorylation site. Mol. Cell. Biol. 10:5305-5313.

37. Luria, S., I. Chambers, and P. Berg. 1991. Expression of the type 1 human immunodeficiency virus Nef protein in T cells prevents antigen receptor-mediated induction of interleukin 2 mRNA. Proc. Natl. Acad. Sci. USA 88:5326-5330.

38. Mariani, R. and J. Skowronski. 1993. CD4 down-regulation by *nef* alleles isolated from human immunodeficiency virus type 1-infected individuals. Proc. Natl. Acad. Sci. USA (12):5549-53.

39. Meyaard, L., S. A. Otto, R. R. Jonker, M. J. Mijnster, R. P. M. Keet, and F. Miedema. 1992. Programmed cell death of T cells in HIV-1 infection. Science 257:217-219.

40. Miller, M. D., M. T. Warmerdam, I. Gaston, W. C. Greene, and M. B. Feinberg. 1994. The human immunodeficiency virus-1 *nef* gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages. J. Exp. Med. 179:101-113.

41. Mittler, R. S., B. M. Rankin, and P. A. Kiener. 1991. Physical associations between CD45 and CD4 or CD8 occur as late activation events in antigen receptor-stimulated human T cells. Journal of Immunology 147:3434-3440.

42. Nakayama, T., C. H. June, T. I. Munitz, M. Sheard, S. A. McCarthy, S. O. Sharrow, L. E. Samelson, and A. Singer. 1990. Inhibition of T cell receptor expression and function in immature CD4+CD8+ cells by CD4. Science 249:1558-1561.

43. Nakayama, T., A. Singer, E. D. Hsi, and L. E. Samelson. 1989. Intrathymic signalling in immature CD4+CD8+ thymocytes results in tyrosine phosphorylation of the T-cell receptor z chain. Nature 341:651-654.

44. Nakayama, T., D. L. Wiest, K. M. Abraham, T. I. Munitz, R. M. Perlmutter, and A. Singer. 1993. Decreased signaling competence as a result of receptor overexpression: overexpression of CD4 reduces its ability to activate $p56^{lck}$ tyrosine kinase and to regulate T cell antigen receptor expression in immature CD4+CD8+ thymocytes. Proc. Natl. Acad. Sci. USA 90:10534-10538.

45. Niederman, T. M., J. V. Garcia, W. R. Hastings, S. Luria, and L. Ratner. 1992. Human immunodeficiency virus type 1 Nef protein inhibits NF-κB induction in human T cells. J. Virol. 66:6213-6219.

46. Niederman, T. M., W. R. Hastings, S. Luria, J. C. Bandres, and L. Ratner. 1993. HIV-1 Nef protein inhibits the recruitment of AP-1 DNA-binding activity in human T-cells. Virology 194:338-344.

47. Niederman, T. M., B. J. Thielan, and L. Ratner. 1989. Human immunodeficiency virus type 1 negative factor is a transcriptional silencer. Proc. Natl. Acad. Sci. USA 86:1128-1132.

48. Novak, T. J., D. Farber, D. Leitenberg, S. C. Hong, P. Johnson, and K. Bottomly. 1994. Isoforms of the transmembrane tyrosine phosphatase CD45 differentially affect T cell recognition. Immunity. 1:109-119.

49. O'Rourke, A. M., J. Rogers, and M. F. Mescher. 1990. Activated CD8 binding to class I protein mediated by the T-cell receptor results in signalling. Nature 346:187-189.

50. Oyaizu, N., N. Chirmule, V. S. Kalyanaraman, W. W. Hall, R. A. Good, and S. Pahwa. 1990. Human immunodeficiency virus type 1 envelope glycoprotein gp120 produce immune defects in CD4+ T lymphocytes by inhibiting interleukin-2 mRNA. Proc. Natl. Acad. Sci. USA 87:2379

51. Oyaizu, N., T. W. McCloskey, S. Than, R. Hu, V. S. Kalyanaraman, and S. Pahwa. 1994. Cross-linking of CD4 molecules upregulates Fas antigen expression in lymphocytes by inducing interferon-gamma and tumor necrosis factor-alpha secretion. Blood 84:2622-2631.

52. Pantaleo, G., C. Graziosi, J. F. Demarest, L. Butini, M. Montroni, C. H. Fox, J. M. Orenstein, D. P. Kotler, and A. S. Fauci. 1993. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. Nature 362:355-359.

53. Ray, C. A., R. A. Black, S. R. Kronheim, T. A. Greenstreet, P. R. Sleath, G. S. Salvesen, and P. J. Pickup. 1992. Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the IL-1b-converting enzyme. Cell 69:597-604.

54. Saizawa, K., J. Rojo, and C. A. J. Janeway. 1987. Evidence for a physical association of CD4 and the CD3:α:β T cell receptor. Nature 328:260-263.

55. Sakihama, T., A. Smolyar, and E. L. Reinherz. 1995. Oligomerization of CD4 is required for stable binding to class II MHC proteins but not for interaction with HIV gp120. Proc. Natl. Acad. Sci. USA 92:6444-6448.

56. Saksela, K., G. Cheng, and D. Baltimore. 1995. Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef+ viruses but not for down-regulation of CD4. EMBO J. 14:484-491.

57. Salemi, S., A. P. Caporossi, L. Boffa, M. G. Longobardi, and V. Barnaba. 1995. HIV gp120 activates autoreactive CD4-specific T cell responses by unveiling of hidden CD4 peptides during processing. J. Exp. Med. 181:2253-2257.

58. Sawai, E. T., A. Baur, H. Struble, B. M. Peterlin, J. A. Levy, and C. Cheng-Mayer. 1994. Human immunodeficiency virus type 1 Nef associates with a cellular serine kinase in T lymphocytes. Proc. Natl. Acad. Sci. USA 91:1539-1543.

59. Skowronski, J., D. Parks, and R. Mariani. 1993. Altered T cell activation and development in transgenic mice expressing the HIV-1 *nef* gene. EMBO J. 12:703-713.

60. Spina, C. A., T. J. Kwoh, M. Y. Chowers, J. C. Guatelli, and D. D. Richman. 1994. The importance of *nef* in the induction of human immunodeficiency virus type 1 replication from primary quiescent CD4 lymphocytes. J. Exp. Med. 179:115-123.

61. Thome, M., P. Duplay, M. Guttinger, and O. Acuto. 1995. Syk and ZAP-70 mediates recruitement of $p56^{lck}$/CD4 to the activated T cell receptor/CD3/z complex. J. Exp. Med. 181:1997-2006.

62. Veillette, A., M. A. Bookman, E. M. Horak, L. E. Samelson, and J. B. Bolen. 1989. Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase $p56^{lck}$. Nature 338:257

63. Wei, X., S. K. Ghosh, M. E. Taylor, V. A. Johnson, E. A. Emini, P. Deutsch, J. D. Lifson, S. Bonhoeffer, M. A. Nowak, and B. H. Hahn. 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature 373:117-122.

64. Westendorp, M. O., R. Frank, C. Ochsenbauer, K. Stricker, J. Dhein, H. Walczak, K. M. Debatin, and P. H. Krammer. 1995. Sensitization of T cells to CD95 mediated apoptosis by HIV-1 Tat and gp120. Nature 375:497-500.

65. Xu, H. and D. R. Littman. 1993. A kinase-independent function of Lck in potentiating antigen-specific T cell activation. Cell 74:633-643.

66. Zazopoulos, E. and W. A. Haseltine. 1993. Effect of *nef* alleles on replication of human immunodeficiency virus type 1. Virology 194:20-27.

67. Zerbib, A. C., A. B. Reske-Kunz, P. Lock, and R. P. Sekaly. 1994. CD4-mediated enhancement or inhibition of T cell activation does not require the CD4:$p56^{lck}$ association. Journal of Experimental Medicine 179:1973-1983.

CLAIMS TO ORIGINAL RESEARCH

Demonstration that the association of lck with CD4 can inhibit proliferation induced through the TcR and that co-aggregation of the CD4/lck association with the TcR is required to initiate T cell activation.

Demonstration that the extracellular domain of CD4 modulates T cell activation independently of its interaction with MHC class II molecules and its association with lck.

Demonstration that inhibition of antigenic stimulation by gp120 does not require association of CD4 with lck, activation of the CD4-associated lck or endocytosis of CD4.

Demonstration that the association of lck with CD4 modulates HIV-1 replication by a post-transcriptional mechanism.

Demonstration that the HIV Nef protein down regulates CD4 expression by two mechanisms: degradation of cell surface CD4 in the lysosomal compartment and inhibition at the level of synthesis of CD4 molecules.

Identification of the amino acids and structures of the cytoplasmic domain of CD4 involved in down regulation of cell surface expression of CD4 induced by Nef.

Dissociation of the pathways of degradation of CD4 induced by PMA and Nef. Demonstration that Nef does not dissociates lck from CD4 to induce internalization of CD4.

Demonstration that the dissociation of lck from CD4 in HIV infected cells is not due to the presence of Nef.