# THE gp23/45/90 THYMIC ADHESION COMPLEX.

by

CLEMENT COUTURE

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Department of Medicine
Division of Experimental Medicine
McGill University
Montreal, Canada

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**ghItlh vlghltlhta'bogh DalaD'a'**

*- Old Klingon Proverb*

## ABSTRACT

Lympho-stromal interactions have been shown to play a vital role in the control of all aspects of intrathymic T cell development. As a consequence, the molecules involved in these interactions and the intracellular signals triggered in developing T cells during their interactions with the stromal elements of the thymus have become the subject of intense investigation. One model of such lympho-stromal interaction is the adhesion of $CD4^+CD8^+$ thymocytes to the E-5 thymic medullary epithelial cell line. In this work, we developed a solid-phase adhesion assay to demonstrate that the interaction of $CD4^+CD8^+$ thymocytes with E-5 cells is mediated by a novel adhesion molecule, the gp23/45 complex, expressed by the epithelial cells. This adhesion molecule is physically associated with a third subunit, gp90, which is not required for thymocyte adhesion. However, thymocyte adhesion to E-5 cells via the gp23/45 adhesion molecule induces the phosphorylation of gp90 by a protein tyrosine kinase associated with the adhesion complex. These observations demonstrate that lympho-stromal interactions can result in thymic stromal cell activation and provide strong support to the concept that thymocytes may regulate the activity of thymic stromal cells.

## RESUME

Il est présentement reconnu que les interactions lympho-stromales jouent un rôle vital dans la régulation de tous les événements associés au développement des lymphocytes T dans le thymus. En conséquence, les molécules impliquées dans ces interactions, de même que les signaux intracellulaires induits chez les thymocytes lors de ces interactions avec les éléments stromaux du thymus, font l'objet d'une intense investigation. Un modèle d'interaction lympho-stromale est l'adhérence des thymocytes $CD4^+CD8^+$ aux cellules épithéliale médullaire thymiques de la lignée E-5. Dans ce travail, nous avons développé un test d'adhérence en phase solide pour démontrer que l'adhérence des thymocytes $CD4^+CD8^+$ aux cellules E-5 est médiée par une nouvelle molécule d'adhérence, le dimère gp23/45, qui est exprimé par les cellules épithéliales. Cette molécule d'adhérence est associée à une troisième sous-unité, la gp90, qui n'est pas nécessaire pour l'adhérence des thymocytes. Cependant, l'adhérence des thymocytes aux cellules E-5 via le dimère gp23/45 induit la phosphorylation de la gp90 par une tyrosine kinase qui est associée au complexe d'adhérence. Ces observations démontrent que les interactions lympho-stromales peuvent induire l'activation des cellules stromales et supportent le concept que les thymocytes puissent réguler l'activité des cellules du stroma thymique.

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## LIST OF ABBREVIATIONS

The abbreviations listed in this sections are in accordance with the list of standard abbreviations published in the *Journal of Immunology*.

| | | |
|---|---|---|
| ATP | : | Adenosine triphosphate |
| BSA | : | Bovine serum albumine |
| cDNA | : | complementary DNA |
| Ci | : | Currie |
| CPM | : | Counts per minute |
| $^{51}Cr$ | : | $^{51}$Chromium |
| CTL | : | Cytotoxic T lymphocyte |
| DAF | : | Decay-accelerating factor |
| DAG | : | Diacylglycerol |
| DEAE | : | Diethylaminoethyl |
| DOC | : | Sodium deoxycholate |
| DNA | : | Deoxyribonucleic acid |
| E-5gp | : | E-5 glycoprotein extract |
| EDTA | : | Ethylenediaminetetraacetic acid |
| EGF | : | Epidermal growth factor |
| $F(ab)'_2$ | : | Divalent antigen-binding fragment |
| FCS | : | Fetal calf serum |
| FTOC | : | Fetal thymic organ culture |
| *g* | : | Unit of gravity |
| GPI | : | Glycosyl-phosphatidylinositol |
| HBSS | : | Hanks' balanced salt solution |
| HIV | : | Human immunodeficiency virus |
| HPLC | : | High performance liquid chromatography |
| ICAM | : | Intercellular adhesion molecule |
| IFN | : | Interferon |
| Ig | : | Immunoglobulin |
| IL | : | Interleukin |
| $IP_3$ | : | Inositol 1,4,5-triphosphate |
| kD | : | Kilo dalton |

| | | |
|---|---|---|
| LAK | : | Lymphokine-activated killer cells |
| LFA | : | Lymphocyte function-associated antigen |
| LPS | : | Lipopolysaccharide |
| mAb | : | Monoclonal antibody |
| MAG | : | Myelin-associated glycoprotein |
| MEM | : | Minimun essential medium |
| MHC | : | Major histocompatibility complex |
| MLR | : | Mixed lymphocyte reaction |
| MNNG | : | n-methyl-n'-nitro-n-nitrosoguanidine |
| mRNA | : | Messenger RNA. |
| *n* | : | Number in study or group |
| NCAM | : | Neural cell adhesion molecule |
| ND | : | Not determined |
| NK cell | : | Natural killer cell |
| NP-40 | : | Nonidet P-40 |
| O.D. | : | Optical density |
| *p* | : | Probability |
| PAGE | : | Polyacrylamide gel electrophoresis |
| PBS | : | Phosphate-buffered saline |
| PDGF | : | Platelet-derived growth factor |
| PHA | : | Phytohemagglutinin |
| PI | : | Phosphatidyl inositol |
| $PIP_2$ | : | Phosphatidylinositol 4,5-biphosphate |
| PLC | : | Phospholipase C |
| PKC | : | Protein kinase C |
| PMA | : | Phorbol myristate acetate |
| PMSF | : | Phenylmethylsulfonyl fluoride |
| PSI | : | Pounds per square inch |
| PTK | : | Protein tyrosine kinase |
| PVC | : | Polyvinyl chloride |
| R | : | Receptor (e.g., IL-2R) |
| RNA | : | Ribonucleic acid |
| SDS | : | Sodium dodecyl sulfate |
| SRBC | : | Sheep red blood cells |
| SV40 | : | Simian virus 40 |

| | | |
|---|---|---|
| TAP | : | T cell-activating protein |
| TBS | : | Tris-buffered saline |
| TCA | : | Trichloroacetic acid |
| TCR | : | T cell receptor for antigen |
| Th | : | T helper cell |
| TNF | : | Tumor necrosis factor |
| Tris | : | Tris(hydroxymethyl)aminomethane |
| Ts | : | T suppressor cell |
| TSTGF | : | Thymic stroma-derived T-cell growth factor |

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## PROLOGUE

This thesis describes the identification of a novel thymic epithelial adhesion molecule and its involvement in the stimulation of thymic stromal cells by thymocyte contact. I have chosen the option provided in Section 7 of the Guidelines Concerning Thesis Preparation of the Faculty of Graduate Studies and Research of McGill University, which reads as follows:

"The candidate has the option, subject to the approval of the Department of including as part of the thesis the text, or duplicated published text, of an original paper, or papers. In this case, the thesis must still conform to all other requirements explained in Guidelines Concerning Thesis Preparation. Additional material (procedural and design data as well as descriptions of equipment) must be provided 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. It must include a general abstract, a full introduction and literature review and a final overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interest of cohesion.

E-5 adhesion molecule (chapter 3) and Mr. Gustavo Amarante-Mendes, a Ph.D. student in our laboratory, whose project included the study of the signal transduction events triggered in thymocytes after contact with E-5 cells. Mr. Mendes helped in the design and execution of some phosphorylation experiments, common to both projects, which are described in chapter 4, sections 4.41 and 4.42.

# CHAPTER 1

## REVIEW OF THE LITERATURE

Over the last few years, the obligatory participation of lympho-stromal interactions in intrathymic T cell differentiation has been recognized. Several molecules involved in these intercellular interactions have been characterized, and much attention is now paid to the molecular activation events triggered during these interactions. In this chapter, we will discuss the machinery of lympho-stromal interactions and their role in the regulation of T cell development. In this regard, several experimental models will be presented, including the E-5 model of lympho-stromal interactions which was developed by this laboratory.

This thesis is concerned with the identification and the characterization of a novel adhesion molecule, the gp23/45/90 adhesion complex, which is expressed by mouse thymic epithelial cells and is involved in thymocyte adhesion. In the first sections of this introduction, we will describe the anatomy of the adult murine thymus as well the major events of intrathymic T cell differentiation. We will then discuss experimental evidence that suggest that these events may be triggered and controlled by the interactions of developing thymocytes, at successive stages of development, with different thymic stromal cells. Hence, the molecules mediating these lympho-stromal interactions must play a pivotal role in T cell development, possibly by generating intracellular signals which are required for thymocyte differentiation.

The second part of this introduction will therefore provide a review of the major intercellular interaction mechanisms, such as adhesion molecules, known to be active in the immune system. The structure and the role of these molecules will be explored, with a special emphasis on their signal transduction properties. Although most of these accessory molecules have been studied using peripheral mature T cells, they are also expressed by thymocytes and thymic stromal cells, where their function in T cell development is largely unknown.

## 1.1 Anatomy of the adult murine thymus.

The thymus is the primary lymphoid organ responsible for the development of T lymphocytes. The functions of the thymus include the reception and proliferation of committed hematopoietic progenitors (pre-T cells), the elimination of auto-reactive T cells, the selection and maturation of MHC-restricted T cells, and their selective release to the periphery. In this section, we will see that the thymus is heterogenous in nature. It is composed of distinct cell types, which in turn form the distinct thymic microenvironments which are successively encountered by developing thymocytes.

### 1.11 Thymic epithelial cells

The thymus consists of two separate lobes, each organized into two distinct compartments: an outer, or cortical region of densely packed cells and a more sparsely populated, inner medullary area. By and large, the thymus is composed of epithelial cells originating from both the ectoderm of the third brachial cleft and the endoderm of the third pharyngial pouch (Auerbach, 1961, Cordier and Haumont, 1980, Owen and Jenkinson, 1984, reviewed by van Ewijk, 1984).

The majority of stromal (non-lymphoid) cells in the cortex are "type-I" epithelial cells with long processes

interconnected by the means of desmosomes, thus forming a tridimensional network through which thymocytes are interspersed (Nabarra and Andrianarison, 1987 and van Ewijk, 1988, reviewed by van Ewijk, 1991). The medulla contains "type-II" epithelial cells, which are more voluminous, oval-shaped cells, with shorter cytoplasmic extensions. Cortical and medullary epithelial cells are also distinct in the expression of several cell surface antigens. Cortical epithelial cells express high levels of class II MHC products (Ia antigens), H-2D, but no H-2K (class I MHC) determinants, whereas medullary epithelial cells express high levels of all three antigens (van Ewijk et al., 1980 and Barclay and Mayrhofer, 1981). Cortical and medullary epithelial cells can also be distinguished on the basis of their distinct reactivities to various monoclonal antibodies. For example, cortical epithelial cells react with the ER-TR4 mAb, developed by van Ewijk's group (van Vliet et al., 1984, 1985), and also express the B-cell lineage antigen defined by mAb 6C3 (Adkins et al., 1988). Medullary epithelial cells, on the other hand, react with the ER-TR5 mAb (van Vliet et al., 1984, 1985) and expressed the newly identified G8.8 antigen, a glycoprotein also expressed by subcapsular epithelial cells (Farr et al., 1991). Finally, the cortical and medullary epithelial heterogeneity has also been defined on the basis of differential keratin expression (Savino and Dardenne, 1985 and

Farr and Braddy, 1989) as well as distinct lectin reactivity (Farr and Anderson, 1985).

In the cortex, some epithelial cells in the subcapsular cortical region are arranged in "baskets" filled with up to 200 thymocytes. These structures, called thymic nurse cells or TNCs, were discovered by Wekerle in 1980 (Wekerle and Ketelson, 1980 and Wekerle et al., 1980). Their phenotype is similar to that of cortical epithelial cells (van Vliet et al., 1984), although they have been found to react with the ER-TR7 mAb (Defresne, 1986), which normally recognizes fibroblasts. The TNC are believed to constitute a distinct thymic microenvironment (Wekerle and Ketelson, 1980 and Andrews and Boyd, 1985).

## 1.12 Macrophages and dendritic cells

Macrophages and dendritic cells both originate from the mesoderm, and colonize the fetal murine thymus between day 10 and 11 (Moore and Owen, 1967 and Jotereau et al., 1987). Both cell types are present in the cortex, but they appear to be more abundant in the medulla (Duijvestijn and Hoefsmit, 1981 and Mueller et al., 1987). Macrophages and dendritic cells both express class I MHC antigens (H-2K and H-2D; Robinson and Jordan 1983 and van Ewijk, 1984); however, only dendritic cells were found to express class II MHC products (Ia;

expressed by thymocytes and mature T cells. The developmentally regulated expression of these antigens by thymocytes as they enter into and migrate within the thymus can therefore be monitored, and hence, thymocyte subpopulations can be identified. In this section, we will discuss the major events of T cell development, as they can be monitored by the expression of the αβ T cell antigen receptor/CD3 complex (TCR/CD3, section 1.511), and the CD4 and CD8 (section 1.512) surface markers.

## 1.21 Early T cells.

Historically, the cortical $CD4^-CD8^-$ (double negative) population, representing 4-5% of all thymocytes, has been considered to be the most immature population in the murine adult thymus. In fetal thymic organ culture, these cells were shown to be the precursors of more mature thymocytes (Ceredig et al., 1983). Last year, an even earlier precursor, preceding the double negative stage and capable to give rise to double negative and more mature thymocytes, was identified in the adult thymus and showed to express low levels of CD4 (Wu et al., 1991). Although $CD4^-CD8^-$ thymocytes generally show no surface expression of the TCR/CD3 complex (Bluestone et al., 1987), it is now evident that the majority of TCR gene rearrangements occurs during this stage (Raulet et al., 1985). More recently, however, it was shown that certain

subpopulations of double negative thymocytes express the αβ TCR at the cell surface (Pearse et al., 1988).

The double negative stage of thymocyte development is also characterized by the transient expression of the IL-2 receptor (IL-2R), which occurs before the surface expression of either CD4 or CD8 (Pearse et al., 1989). IL-2R expression marks a burst of cell proliferation and TCR gene rearrangement, as well as the loss of thymus reconstitution ability.

## 1.22 Double positive ($CD4^+CD8^+$) thymocytes and negative selection

It is now accepted that $CD4^-CD8^-$ thymocytes give rise to the more mature $CD4^+CD8^+$ (double positive) thymocytes. Several years ago, an intermediate between double negative and double positive was defined, which express only CD8 (i.e. $CD4^-CD8^+CD3^-$ ; Bluestone et al., 1987, Paterson and Williams, 1987 and MacDonald et al., 1988a). It was therefore believed that double negative thymocytes become double positive by expressing the CD8 antigen first and then CD4. More recently, however, an equivalent intermediate population was identified which express only CD4 (i.e. $CD4^+CD8^-CD3^-$). This population was identified in both irradiation chimeras (Matsumoto et al., 1989 and Arase et al., 1990) and normal mice (Hugo et al., 1990 and 1991). Each of these "intermediate" populations can

give rise to double positive thymocytes and subsequently to more mature single positive ($CD4^+CD8^-CD3^+$ or $CD4^-CD8^+CD3^+$) populations (Guidos et al., 1989 and Hugo et al., 1990).

Double positive thymocytes are found largely in the thymic cortex and they represent the predominant cell in the thymus (75-80% of all thymocytes). In addition to CD4 and CD8 surface expression, about 50% of double positive thymocytes bear surface TCR/CD3, although at much lower concentrations than peripheral T cells (Roehm et al., 1984). These $TCR/CD3^+$ double positives are known to give rise to the more mature single positive ($CD4^-CD8^+$ or $CD4^+CD8^-$) thymocytes (Smith, 1987, MacDonald et al., 1988b, Kisielow 1988 and Sha et al., 1988a), which are found in the medulla and are phenotypically almost indistinguishable from peripheral T cells, bearing high levels of TCR/CD3.

The expression of a randomly rearranged TCR (section 1.5111) by cortical $CD4^+CD8^+$ thymocytes creates the need for a selection mechanism which will allow the further development (positive selection) of only those thymocytes bearing self MHC (major histocompatibility complex)-restricted TCRs, and the elimination of all other thymocytes. As a result, the vast majority of double positive thymocytes dies within the thymus either because they have failed to express a functional TCR at their surface, or because their TCR is reactive to self

antigens (reviewed by Sprent and Webb, 1987 and by Blackman et al., 1990). This process, aimed to eliminate self-reactive TCR/CD3$^+$ thymocytes, is called negative selection and has been shown to occur mainly through clonal deletion, both in normal mice (Kappler et al., 1987, 1988, Fowlkes et al., 1988 and MacDonald et al., 1988b) and in transgenic mice models (Sha et al., 1988b and Kisielow et al., 1988). The functional inactivation (or clonal anergy) of self-reactive thymocytes has also been observed in the thymus and is postulated to be another means to tolerize thymocytes reactive to self antigens (reviewed by Ramsdell and Fowlkes, 1990).

## 1.23 Positive selection and single positive thymocytes.

While negative selection is thought to be controlled by MHC$^+$ cortical dendritic cells (Speiser et al., 1989), the positive selection events, which lead to the preferential differentiation of TCR$^+$ double positive thymocytes that have restriction specificity for self-MHC glycoproteins, might be under the control of MHC$^+$ cortical epithelial cells (Speiser et al., 1989 and Benoist and Mathis, 1989). This process is thought to involve the participation of the TCR/CD3 complex as well as stromal MHC molecules. According to this instructional model, thymocytes bearing a TCR restricted to class II MHC products will down regulate the expression of CD8 to become CD4$^+$CD8$^-$, while a class I MHC-restricted TCR will cause the

down regulation of CD4, leading to the production of $CD4^-CD8^+$ thymocytes (Teh et al., 1988, Robey et al., 1991 and Borgulya et al., 1991). Both of these single positive populations represent 10-15% of all thymocytes and are found in the medulla where they will acquire the functional maturity of helper ($CD4^+CD8^-$) and cytotoxic/supressor ($CD4^-CD8^+$) T cells before migrating to the periphery.

## 1.3 Lympho-stromal interactions in T cell development

The obligatory involvement of cell-cell contact between thymocytes and the stromal elements of the thymus (lympho-stromal interactions) in T cell development has been well demonstrated by Andrews et al. (1985), who showed that the humoral function of thymic stromal cells was unable, by itself, to induce all the events required for T cell differentiation. The identification and isolation of several types of lympho-stromal complexes from adult and newborn mouse thymus is consistent with the successive formation of such complexes *in vivo* (Kyewski, 1987). Hence, it was established that immature thymocytes form complexes first with cortical $Ia^-$ macrophages, then with $Ia^+$ cortical epithelial cells (including thymic nurse cells), and finally with $Ia^+$ medullary dendritic cells (Kyewski, 1987).

The development of thymic stromal cell lines by several laboratories has provided the most direct evidence that lympho-stromal interactions are involved in T cell differentiation. Indeed, several of these cell lines can interact with immature thymocytes and induce *in vitro* some of the events known to be required for *in vivo* T cell development. Hence, transformed human cortical epithelial cells have been found to form complexes with thymocytes and secrete hemopoietic growth factors (Mizutani et al., 1987). Similarly, Barton Haynes' laboratory has shown that cultures of purified human thymic epithelial cells can induce the proliferation of both immature (double negative; Denning et al., 1988a) and mature (Denning et al., 1987) thymocytes. Unfortunately, the full characterization of the epithelial cells inducing these events has not been done. Mouse and rat thymic epithelial cell lines, as well as a rat fibroblast cell line, were also found to induce mouse thymocyte activation by direct cell-cell contact (Savion et al., 1989).

Convincing evidence for the direct role of lympho-stromal interactions in T cell differentiation has been provided by Tatsumi et al. (1990), who developed a thymic stromal cell clone (MRL104.8a) capable of inducing the differentiation of $CD3^-CD4^-CD8^-$ murine thymocytes into more mature phenotypes. Similar observations have been reported by two different groups. First, Brightman et al. (1989) reported that a murine

T lymphoid cell line could be induced, by contact with an uncharacterized thymic stromal cell line, to express the CD4 and Thy-1 antigens (sections 1.512 and 1.521, respectively). Second, Palacios et al. (1989) have demonstrated that the differentiation of $TCR^-$ pro-thymocytes into $TCR^+$ cells, which cannot be obtained by any soluble factor, can be induced by contact with the ET thymic epithelial clone *in vitro*. Finally, the ability of certain thymic stromal cell clones to induce the death of immature thymocytes (Kosaka et al., 1989, Matsubara et al., 1990 and Hiramine et al., 1990) supports the view that lympho-stromal interactions might be involved in the processes of negative and positive selection. In one of these models, a thymic stromal cell clone has been shown to secrete the cytokine TSTGF (Thymic stroma-derived T-cell growth factor), which promotes the proliferation of antigen-specific T cell clones in absence of antigen or IL-2. When these $Ia^+$ stromal cells were pulsed with the relevant antigen, they induced a TSTGF-dependent lethal growth arrest of the T cell clones bearing a TCR specific for that antigen (Kosaka et al., 1989). Although the thymic stromal cell clone has not been well characterized, it might provide a valid model for clonal elimination in the thymus.

The results obtained with these approaches are all consistant with an obligatory role of lympho-stromal interactions at least in some aspects of T cell development.

They also indicate that these contacts result in the delivery of "signals", from the stromal cells to the developing T cells, which induce and control thymocyte differentiation. It is therefore vital to further characterize these interactions. What are the molecules involved in such interactions? How are signals delivered to the thymocytes during contact with thymic stromal cells? Does the contact affect only thymocytes or does it also regulate the activity of the thymic stromal cells involved?

## 1.4 The E-5 model for lympho-stromal interactions

In an attempt to answer some of these questions, our laboratory has developed a mouse thymic stromal cell line named E-5, originating from a primary culture of thymic stromal cells from C57Bl/6 mice, immortalized with a chemical carcinogen. (Potworowski et al., 1986a). Their phenotype is characteristic of medullary epithelial cells: they express cytokeratin and react with medullary epithelial cell-specific monoclonal antibodies ERTR5 and ERTR6 (van Vliet et al., 1984). It was shown, using an *in vitro* adhesion assay, that E-5 cells were capable to interact with mouse thymocytes (Potworowski et al., 1986b) and with the Ti-6 lymphoma cell line (Hugo et al., 1989). A further characterization of E-5 adhering thymocytes revealed that they were mainly blastic $CD4^+CD8^+$ and that they expressed low levels of the CD3/TCR

complex (Hugo and Potworowski, 1990). The observation that after an initial adhesion with E-5 cells, 50% of adhered thymocytes spontaneously detached from the epithelial cells and irreversibly lost the ability to bind to fresh E-5 cells was interpreted as an indication that this interaction represented a discrete stage of thymocyte differentiation (Hugo and Potworowski, 1989). However, adhesion to E-5 induced no thymocyte proliferation (Hugo and Potworowski, 1990), and only a marginal down regulation of the CD8 surface antigen (reviewed by Potworowski et al., 1989). Furthermore, the fact that thymocyte adhesion to E-5 was not dependent on the H-2 (class I MHC) haplotype of the thymocytes led to the conclusion that the involvement of E-5-like interactions in negative or positive selection of thymocytes was most unlikely (Hugo et al., 1988).

Although E-5 interaction with $CD4^+CD8^+$ thymocytes did not appear, *a priori*, to induce any significant modification to adhering thymocytes, the preliminary characterization of the interaction itself uncovered the existence of a new type of cell-cell interaction (reviewed by Potworowski et al., 1989, and P. Hugo, Ph.D. thesis). Indeed, the interaction of E-5 with $CD4^+CD8^+$ thymocytes or Ti-6 cells is effective at both 37°C and 4°C, even in absence of divalent cations. The E-5 adhesion molecule mediating this interaction was postulated to be a trypsin-sensitive glycoprotein (Potworowski et al.,

1986b, Hugo et al., 1989 and P. Hugo Ph.D. thesis) which binds to a non-homotypic protein receptor expressed by thymocytes and Ti-6 cells. The glycosylated moiety of the E-5 adhesion molecule is not required for thymocyte adhesion.

In addition to these observations, it was found that thymocyte adhesion to E-5 cells resulted in the activation of non-adhering thymocytes (Hugo and Potworowski, 1990), which were found to be more reactive to polyclonal T cell activators and IL-2. Since E-5 cell supernatant could not induce these events, we postulate that the adhering thymocytes, which do not appear to be affected by their interaction with E-5 cells, could trigger or regulate the secretion of a soluble factor by the epithelial cell line. Indeed, preliminary experiments from our laboratory demonstrated that thymocyte contact could induce the secretion of IL-1 by E-5 cells. The interaction of E-5 cells with $CD4^+CD8^+$ thymocytes might therefore provide a valid model for the study of the regulation of thymic stromal cell activity by thymocyte contact.

In order to gain an insight into the possible mechanisms by which $CD4^+CD8^+$ thymocytes might interact with E-5 cells, we will now review the major molecular systems that thymocytes and T lymphocytes use to interact with neighbouring cells and which are responsible for both their development and their function. A comparison of the properties of these adhesion

systems with those of the E-5/thymocyte interaction outlined previously should allow us to eliminate certain or all of these systems as being involved in our E-5 model. Also, a special emphasis will be placed on the mechanisms by which intracellular signals can be generated as a result of several types of lympho-stromal interactions.

## 1.5 Molecules expressed by T cells and developing thymocytes

This section is a review of the major cell-cell interaction systems used by both thymocytes and mature T cells. It is important to note that these systems have been studied with the pre-conception that the role of these interactions was to regulate the development and the activity of T cells. Consequently, little attention has been given to the effects that T cells or thymocytes may have on interacting stromal cells, and there is only scanty evidence, even today, that lympho-stromal interactions can result in stromal cell activation (see chapter 4). For example, it was recently observed that thymocytes could induce human thymic epithelial cells to secrete IL-1 (Le et al., 1990). Although the authors have determined that this stromal cell induction was dependent upon the CD2/LFA-3 cellular adhesion pathway (see section 1.522), the signaling events triggered by this interaction have not been studied. For these reasons, we will concentrate our attention to the T cell and thymocyte activation events

trigered by cell-cell interactions, and postulate that similar events might be operative in thymic stromal cell activation.

## 1.51 The antigen recognition system

Unlike the antigen receptor expressed by B lymphocytes, the T cell antigen receptor (TCR) does not recognize antigens in soluble forms, but rather antigenic peptides presented in association with the products of the major histocompatibility complex (MHC). This recognition is provided by the TCR, which binds the antigen/MHC complex expressed by antigen presenting cells (Inaba and Steinman, 1985 and Spits et al., 1986). This interaction is stabilized by the simultaneous interaction of either CD4 or CD8 with non-polymorphic regions of MHC class II or class I products, respectively. For this reason, we will consider both TCR and CD4/CD8 as being part of the antigen recognition system.

### 1.511 The T cell receptor (TCR)/CD3 complex

#### 1.5111 Structure and function.

Antigen recognition by T lymphocytes is mediated by the T cell receptor (TCR, formerly named Ti). This receptor was originally identified by anti-idiotypic antibodies raised against T cell tumors (Allison et al., 1982) or antigen-

specific T cell clones (Meuer et al., 1983 and Haskins et al., 1983), and later by molecular techniques (Yanagi et al., 1984 and Hedrick et al., 1984). At least two distinct types of antigen receptors have been identified on the surface of T cells. The αβTCR (Allison et al., 1982 and Samelson et al., 1983) and the γδTCR (Bank et al., 1986, Brenner et al., 1987, and Borst et al., 1987) are each composed of two disulfide-linked and highly polymorphic glycoproteins, namely α (40 kD), ß (45 kD), γ (21 kD), and δ(28 kD) (reviewed by Marrack and Kappler, 1987, Allison and Lanier, 1987 and Brenner et al., 1988). All four TCR chains are similar to immunoglobulins in their overall primary structure, gene organisation and rearrangement patterns (reviewed by Clevers et al., 1988). The TCRα locus is composed of approximately 50 variable (V) regions, 50 joining (J) regions, and one constant (C) segment, while the TCRβ locus is composed of 70 V, 2 diversity (D), 13 J, and 2 C segments (reviewed by Toyonaga and Mak, 1987, and Davis and Bjorkman, 1988). The combinatorial association of different V-D-J-C segments of both α and β proteins, as well as germ-line diversity can give rise to up to $10^7$ different TCRs with extensive clonal diversity. The vast majority of T cells in normal mice and humans bear the αβTCR, specific for peptide fragments of foreign antigens presented in the context of self MHC proteins (reviewed by Marrack and Kappler, 1987 and Davis and Bjorkman, 1988). Cell fusion experiments (Yague et al., 1985) and transfection of α- and β- chain cDNAs

between different T-cell clones (Saito et al., 1987) confirmed that the α and β heterodimer confers both antigen and self-MHC specificity (reviewed by Hedrick, 1988 and Fink et al., 1988).

The TCRγδ subset comprises 1 to 10% of the cells in the thymus and in the peripheral lymphoid organs (Brenner et al., 1987, Bluestone et al., 1987 and Maeda et al., 1987), but represent the major T lymphocyte subset of the epidermis (Stingl et al., 1987 and Koning et al., 1987) and of the intestinal epithelium (intraepithelial lymphocytes) in the mouse (Bonneville et al., 1988 and Goodman and Lefrançois, 1988). These observations suggested that some γδTCR lymphocytes may be involved in the surveillance of a variety of epithelia (Janeway, 1988). The TCRγδ gene segments have more limited V region diversity than the αβ genes, but a more extensive variability of the J region (Takihara et al., 1988). The specificity of γδ T cells is not completely understood. The description of a γδTCR-bearing mouse T cell line specific for a MHC allele suggested that γδ T cells may also recognize foreign peptides in association to MHC products (Matis et al., 1987).

Because each of the four TCR polypeptides contain a very short cytoplasmic domain of up to 12 amino acids (Hedrick et al., 1984 and Saito et al., 1984), the TCR is dependent on its association with the CD3 (formerly known as T3) complex for

TCR-driven signal transduction. This invariant CD3 complex was first described as a human T cell specific marker recognized by mAb OKT3 (Kung et al., 1979). Subsequent immunochemical and biochemical studies resulted in the identification and characterization of the polypeptides that comprise the CD3 complex in both murine (Allison and Lanier, 1985, Samelson et al., 1985 and Oettgen et al., 1986) and human (Borst et al., 1983, Oettgen et al., 1984 and Brenner et al., 1985) systems. To date, five distinct polypeptides, namely γ (gamma), δ (delta), ε (epsilon), η (eta) and ζ (zeta), have been identified in non covalent association with the clonotypic αβTCR. These proteins have molecular weights varying from 16 to 28 kD.

The CD3 complex itself is said to be composed of γδε subunits. All three components of CD3 are non-polymorphic proteins that are members of the immunoglobulin supergene family (Gold et al., 1986 and Gold et al., 1987). Both γ and δ subunits contain up to three extracellular N-glycosylation sites, while ε is nonglycosylated (Samelson et al., 1985 and Baniyash et al., 1988a). Recent evidence suggests that two copies of ε are present within a single TCR/CD3 complex (de la Here et al., 1991), and that both ε and γ subunits can inhibit the pre-golgi degradation of the other (more labile) subunits, therefore allowing only the complete and functional receptors to reach the cell surface (Wileman et al., 1990). CD3

association is non-covalent and is highly dependent upon the presence of charged amino acids within the transmembrane domain of the TCRβ chain. In fact, it was found that Lys 271 of the β subunit transmembrane domain was required for CD3 association, but not for TCRαβ dimerization (Alcover et al., 1990).

The $\gamma\delta\epsilon_2$ CD3 complex is associated with the ζ chain, which can be coimmunoprecipitated by anti-CD3 or anti-TCR antibodies, under appropriate conditions (Weissman et al., 1986 and Samelson et al., 1986). The gene for the ζ chain, located on chromosome 1 in both human and mice (Weissman et al., 1988a and Baniyash et al., 1989), encodes a 16 kD nonglycosylated protein unrelated to the other subunits of the CD3 complex. The ζ polypeptide is a transmembrane protein composed of a large intracytoplasmic domain (113 amino acids) and a small extracellular portion (9 amino acids; Weissman et al., 1988a, 1988b). The ζ chain appears to be unrelated in sequence and structure to the CD3 chains. About 90% of ζ is found as a disulfide-linked homodimer, while the remaining 10% forms a heterodimer with the 22 kD η subunit, which suggested the existance of two distinct classes of TCR/CD3 complexes (Baniyash et al., 1988a, Mercep et al., 1988). The ζ and η subunits appeared distinct by aminoacid composition and preliminary peptide mapping (Orloff et al., 1989). However, antisera raised against certain peptides of the ζ chain also

reacted with η, suggesting at least the presence of common epitopes (Orloff et al., 1989). This apparent contradiction was solved by the cloning of the murine η chain, which showed that η and ζ were identical through amino acid 122 of each mature protein, but different beyond this point, yielding cytoplasmic domains of very different lengths (113 and 155 amino acids for ζ and η, respectively; Jin et al., 1990). It was therefore concluded that ζ and η proteins were produced by alternative splicing of a single gene (Clayton et al., 1991). The ζ chain plays an essential role in the assembly and surface expression on the TCR/CD3 complex. This was shown in murine T hybridoma mutants, where the lack of ζ expression resulted in a very low TCR/CD3 surface expression (Sussman et al., 1988a). Similarly, a human T cell immunodeficiency syndrome characterized by a very low expression of intracellular ζ chains and an almost complete lack of TCR/CD3 surface expression has been described (Alarcon et al., 1988).

## 1.5112 Signal transduction through TCR/CD3

The engagement of the TCR by its specific antigen/MHC complex is known to trigger a cascade of events leading to T cell activation. A number of excellent reviews regarding these events have recently been published (Weiss and Imboden, 1987, Clevers et al., 1988, Crabtree, 1989, Altman et al., 1990a, 1990b, and Rao et al., 1991). Ligand binding to the TCR

results in the activation of two known second messenger-generating pathways. First, the ζ chain of the TCR/CD3 complex is rapidly phosphorylated on tyrosine residues, indicating the activation of a protein tyrosine kinase (PTK; Samelson et al., 1986). Ligand binding also leads to the activation of a phospholipase C (PLC), resulting in hydrolysis of phosphatidylinositol 4,5-biphosphate ($PIP_2$) to yield diacylglycerol (DAG) and inositol 1,4,5-triphosphate ($IP_3$; Imboden and Stobo, 1985 and Imboden et al., 1987). While $IP_3$ can mediate the mobilization of intracellular calcium (Berridge and Irvine, 1984, 1989), DAG can activate the calcium-dependent protein kinase C (PKC; reviewed by Bell, 1986, Kikkawa and Nishizuka, 1986, Berridge, 1987, and Rana and Hokin, 1990). These events can be recreated by calcium ionophores, which trigger calcium entry into the cell, and phorbol esters which can directly activate the PKC (Truneh et al., 1985). These treatments, as well as ligand binding to the TCR, induce the serine phosphorylation of both human and mouse CD3γ chains (Patel et al., 1987 and Davies et al., 1987), which is thought to be mediated by PKC and perhaps other serine kinases (Davies et al., 1987 and Cantrell et al., 1989). This CD3γ phosphorylation generally results in the internalization of the entire TCR/CD3 complex (Krangel, 1987), although the direct involvement of serine phosphorylation by PKC in this phenomenon has been questionned (Minami et al., 1987 and Cantrell et al., 1989).

The predicted protein sequence of the ζ subunit revealed the presence of a consensus sequence (GxGxxGxxxGxxxAxK) for nucleotide binding (Weissman et al., 1988a). This observation is important, because the activation of PLC and the subsequent PiP2 hydrolysis cascade have been shown to be regulated by one or several G proteins in T cells (Mustelin et al., 1986, Sasaki and Hasegawa-Sasaki, 1987, O'Shea et al., 1987 and Davies et al., 1988). The identification and purification of a PLC-stimulating G protein from calf thymocytes is consistant with this hypothesis, although the physical association of ζ with a G protein has never been demonstrated (Wang et al., 1989). However, the deletion of this nucleotide binding region of the ζ chain abolished the TCR-mediated T cell activation as measured by antigen-dependent IL-2 production (Frank et al., 1990). Similarly to ζ, the η chain is also thought to be involved in TCR-mediated phosphatidyl inositol (PI) turnover. A mutant of the 2B4 murine hybridoma expressing normal levels of TCR/CD3, but little or no η, showed no PI turnover in response to antigen or anti-CD3 (Mercep et al., 1988). In such $\eta^-$ cells, however, antigen stimulation resulted in IL-2 production, which suggests that PI turnover and IL-2 production in response to TCR stimulation are not causally related (Mercep et al., 1989).

## 1.5113 The $p59^{fyn}$ protein tyrosine kinase.

Minutes after stimulation of the TCR/CD3 complex, either by antigen or anti-CD3 treatment, the ζ chain becomes phosphorylated on multiple tyrosine residues (Samelson et al., 1986, Patel et al., 1987, and Baniyash et al., 1988b). In fact, the increased tyrosine phosphorylation of a number of unidentified intracellular T cell substrates is the first event detected after TCR/CD3 stimulation, and even precedes PLC activation (June et al., 1990). The importance of PTK activity in the overall process of T cell activation was further demonstrated by the use of PTK inhibitors such as genistein and herbimycin A, which shown that the TCR/CD3-induced PI turnover (and hence PLC activation) was not possible in absence of PTK activation (Mustelin et al., 1990). Since the TCR/CD3 complex primary sequence does not contain any recognizable protein kinase domain, it was concluded that the TCR must be coupled to a non-receptor PTK. One candidate for such a kinase was the CD4/CD8-associated $p56^{lck}$ (see section 1.1123). However, even in systems where CD4 cross-linking (leading to lck activation) was required for activation of a T cell clone, it was noticed that TCR cross-linking was in itself insufficient to trigger lck activation (Glachenhaus et al., 1991). Furthermore, lck activation via CD4 cross-linking was shown to result in a pattern of intracellular substrate tyrosine phosphorylation different

from that resulting from TCR cross-linking (Veillette et al., 1989a and Luo and Sefton, 1990). Finally, the observation that in γδTCR-expressing T cells, also negative for CD4 and CD8, the TCR is still coupled to a PTK, suggests the presence of another TCR-associated PTK (reviewed by Klausner and Samelson, 1991).

The identification of the $p59^{fyn}$ PTK, a member of the src-related protein kinases, as a TCR-associated kinase involved in T cell activation came with the use of mice homozygous for the *lpr* and *gld* mutant genes. These mice exhibit lymphoproliferative syndromes characterized by the expansion of an unusual subset of $Thy\text{-}1^{+}$, $CD4^{-}$, $CD8^{-}$ T cells expressing a mature αβTCR (Katagiri et al., 1988). These abnormal T cells overexpress the fyn protein kinase (Katagiri et al., 1989), show constitutive ζ chain tyrosine phosphorylation (Samelson et al., 1986) and are unresponsive to normal TCR stimulants (Scholtz et al., 1988), possibly because of an ineffective coupling between the TCR and PLC (Coggeshall and Altman, 1989). The most convincing evidence (and the most pertinent to this work) that $p59^{fyn}$ can physically associate to the TCR was provided by Samelson and co-workers, who showed that under certain detergent conditions, $p59^{fyn}$ can be co-immunoprecipitated with the TCR/CD3 complex, using either anti-TCRα, anti-CD3ε or anti-$p59^{fyn}$ antibodies (Samelson et al., 1990a). Most important, this group showed the presence of

PTK activity within these precipitates, resulting in the phosphorylation of CD3ζ and the autophosphorylation of $p59^{fyn}$, which were completely devoid of $p56^{lck}$. Although it still has not been directly shown that TCR stimulation leads to fyn activation, it has been shown that $p59^{fyn}$ overexpression in transgenic mice could increase thymocyte responsiveness to TCR stimulants (Cooke et al., 1991), suggesting that the *fyn* product was involved in the regulation of TCR signalling.

The molecular basis of the TCR/CD3 complex association with $p59^{fyn}$ is still unclear. It was recently found that the ζ chain can be associated with a PTK inducible by TCR stimulation. However, this PTK does not appear to be *fyn* (Chan et al., 1991). Another possibility was brought up by the recent observation that the CD3ϵ intracellular domain could be involved PTK activation during T cell stimulation (Letourneur and Klausner, 1992). At this time, the PTK actually interacting with CD3ϵ is not known.

## 1.512 CD4/CD8

### 1.5121 Structure and function.

CD4 and CD8 are two T cell-specific glycoproteins which seperate most mature T lymphocytes into mutually exclusive subsets: helper T cells generally express CD4 while

suppressive/cytotoxic T cells generally express CD8. Both antigens are members of the immunoglobulin supergene family (Williams and Barclay, 1988). CD4 (previously called L3T4 in the mouse) is a 55-67 kD single chain protein, whereas CD8 can be expressed as a heterodimer of α (34-38 kD, originally called Ly-2 in the mouse) plus β (28-30 kD, Ly-3 in the mouse) subunits, or as an α/α homodimer (reviewed by Littman, 1987 and Parnes, 1989). CD4 or CD8 expression by a T cell correlates to its ability to recognize an antigen in the context of MHC class II or class I, respectively (reviewed by Swain, 1983, and Parnes, 1989). This led to the suggestion that CD4 and CD8 might function to increase the avidity of a T cell for its antigen by binding to MHC class II and I products, respectively (Reinherz et al., 1983, Marrack et al., 1983 and Biddison et al., 1984). Transfection studies have been used to demonstrate that the low affinity binding of CD4 to class II MHC in absence of the TCR/CD3 complex was sufficient to mediate cell-cell adhesion (Doyle and Strominger, 1987). Similarly, Gabert and co-workers were able to demonstrate that the co-transfection of both TCR and CD8 genes was necessary to confer a novel class I-restricted specificity to a CD4+CD8- T helper clone previously restricted to class II MHC (Gabert et al., 1987). Here again, it was concluded that CD8 was necessary to stabilize the TCR/Ag interaction. This notion was supported by the observation that purified class I MHC products incorporated within artificial

membranes could interact with CD8 (Goldstein and Mescher, 1987). Finally, the observations that the TCR/CD3 complex and CD4 or CD8 were co-modulated (internalized) during antigen presentation (Saizawa et al., 1987 and Kupfer and Singer, 1988), and physically associated on activated T cells (Rivas et al., 1988, Anderson et al., 1988, Mittler et al., 1989 and Rojo et al., 1989), together with the observation that CD8 was physically associated with MHC class I antigens on activated T cells (Bushkin et al., 1988), were all consistent with the role of CD4/CD8 in the formation of a high avidity antigen recognition structure.

**1.5122 Signal transduction through CD4/CD8.**

The concept that CD4 and CD8 play an active role in T cell activation solely by strengthening the interaction between a low avidity TCR and its antigen (Shimonkevitz et al., 1985) has been challenged by a series of experiments which now suggest that both CD4 and CD8 can transmit independent signals to the T cells. Among the first evidence of CD4/CD8-dependent signal transduction was the observation that anti-CD4 or anti-CD8 antibodies could inhibit T cell function, even in the absence of CD4/CD8 ligands (Hollander et al., 1981 and Bank and Chess, 1985). These observations led to the suggestion that CD4/CD8 could transduce independent negative signals to

T cells. This model, however, is incompatible with several recent observations (see the following section).

A strong line of evidence for independent CD4/CD8 signal transduction arose from cross-linking studies using various anti-CD4 or anti-CD8 mAbs. Indeed, it was reported that the heterologous cross-linking of the TCR/CD3 complex with CD4 or CD8 molecules was much more effective to activate purified human T cells than TCR/CD3 cross-linking alone, as measured by IL-2 receptor expression, cell proliferation and calcium mobilization studies (Emmrich et al., 1986, 1987, Boyce et al., 1988 and Ledbetter et al., 1988). Furthermore, it was found that antibody-mediated cross-linking of CD4 or CD8 alone could induce the release of calcium from intracellular stores (Ledbetter et al., 1987). On the contrary, a soluble anti-CD4 mAb was found to inhibit the mitogen-induced entry of extracellular calcium, suggesting a functional association of CD4 with calcium channels (Rosoff et al., 1987). Interestingly, the binding of gp120 (the CD4-binding envelope glycoprotein of the HIV virus) also stimulates the release of intracellular calcium, plus PI turnover and IL-2 receptor expression (Kornfeld et al., 1988). Finally, it is worth mentioning that the accessory signal provided by CD8 cross-linking was found to be highly sensitive to H-7, a PKC inhibitor, which suggest that CD8 may be directly or

indirectly coupled to PKC or other kinases (Samstag et al., 1988).

## 1.5123 The p56lck protein tyrosine kinase

$p56^{lck}$ was first characterized in the LSTRA cell line (Casnelli et al., 1982) and its expression is relatively restricted to lymphocytes (Voronova and Sefton, 1986 and Veillette et al., 1987). In mice, *lck* is expressed in T cells and, to a lesser degree, in B cells, while in humans it appears to be specific to T cells (Trevillyan et al., 1986 and Koga et al., 1986, 1988). $p56^{lck}$ (and $p59^{fyn}$) belongs to the *src* family of non-receptor protein tyrosine kinases (Marth et al., 1985, 1987).

The notion of a separate signalling function for CD4 and CD8 was validated by the discovery that both molecules are physically associated with the cytoplasmic tyrosine kinase $p56^{lck}$ (Rudd et al., 1988 and Veillette et al., 1988). The presence of the lck product in CD4/CD8 immunoprecipitates was verified by Western blotting and by its ability to phosphorylate itself as well as exogenous substrates (Barber et al., 1989, Rudd et al., 1989 and Shaw et al., 1989). The absence of tyrosine residues in the cytoplasmic domain of CD4 (Tourvieille et al., 1986) makes it unlikely that this molecule represents an lck substrate, and it was therefore

proposed that CD4 and CD8 may regulate T cell activation by antigen or mitogens via their association with $p56^{lck}$ (Mustelin and Altman, 1989). This association is mediated by the cytoplasmic domains of both CD4 and CD8α (Veillette et al., 1990 and Yao et al., 1990), possibly through short related amino acid sequences located at the COOH-terminal portion of CD4/CD8 (Shaw et al., 1989, 1990 and Yao et al., 1990). These small segments both contain two cysteine residues each, which are essential for association with $p56^{lck}$. A similar cysteine motif, present at the amino-terminal portion of $p56^{lck}$, has been shown to be responsible for the interaction with CD4 and CD8 (Shaw et al., 1990 and Turner et al., 1990). Interestingly, these interacting cystein residues are not involved in disulfide bonds (Shaw et al., 1990).

The hypothesis that the CD4/CD8:$p56^{lck}$ complex could be involved in the delivery of an independent signal was verified by Veillette's group, who showed that CD4 cross-linking triggered the PTK activity of $p56^{lck}$, resulting in the tyrosine phosphorylation of some TCR/CD3 complex components ($\gamma, \delta, \epsilon$ and $\zeta$), as well as $p56^{lck}$ itself (Veillette et al., 1989a, 1989b, and Barber et al., 1989). It is worth noting that CD4 and CD8 are also associated to $p56^{lck}$ in immature $CD4^{+}CD8^{+}$ thymocytes. Furthermore, engagement of CD4 or CD8 at the surface of these thymocytes also results in the rapid activation of $p56^{lck}$ (and in an increased tyrosine phosphorylation of diverse cellular

substrates; Veillette et al., 1989c), suggesting that the signalling functions of CD4/CD8 are operative in the thymus.

In T cell hybridomas, $p56^{lck}$ activity has been shown to increase T cell responsiveness to antigen/MHC (Abraham et al., 1991a). In T cells and T cell lines, the role of CD4/CD8:$p56^{lck}$ signalling in TCR-driven activation has been demonstrated by the use of deletion mutants of CD4 and CD8$\alpha$. These altered molecules were devoid of their C-terminal cystein-containing domains and were therefore unable to associate with $p56^{lck}$ (Zamoyska et al., 1989). Compared to T cells expressing normal CD4 or CD8, cells expressing mutated CD4 or CD8 showed no CD8-mediated $p56^{lck}$ activation and early phosphorylation events (Chalupny et al., 1991) and a dramatic decrease (or complete inhibition) in T cell avtivation by nominal antigens (Glaichenhaus et al., 1991 and Miceli et al., 1991).

As stated earlier, $p56^{lck}$ is a member of the *src* family of protein tyrosine kinases. The members of this family all share structural and functional features which are important for the regulation of their PTK activity. Unlike growth factor receptors that possess PTK activity (such as the EGF receptor or the insulin-like growth factor receptor), the *src*-related proteins do not have transmembrane or extracellular sequences (reviewed by Hunter and Cooper, 1985, Hunter, 1987 and Cantley et al., 1991). However, they are closely associated with the

internal side of the plasma membrane because of posttranslational myristylation at their amino termini (Buss and Sefton, 1985). This property is felt to be critical for the proposed function of these molecules in signal transduction and transformation (Cross et al., 1985, Kamps et al., 1985 and Hunter, 1987). The association of myristylated protein kinases to the plasma membrane may be mediated by recently described membrane receptor known to bind $p60^{v\text{-}src}$ (Resh and Ling, 1990).

The regulation of $p56^{lck}$ activity is also common to all members of the *src* family. Veillette and co-workers showed that CD4 cross-linking results in $p56^{lck}$ activation by a mechanism involving the autophosphorylation of $p56^{lck}$ at residue Tyr 394. The replacement of this tyrosine residue by phenylalanine abolished the ability to activate $p56^{lck}$ by CD4 engagement (Veillette and Fournel, 1990). The same tyrosine residue (394, called the autophosphorylation site) was found to be autophosphorylated by CD8 stimulation (Luo and Sefton, 1990). More recently, Ramer and co-workers reported the biochemical purification of $p56^{lck}$. The purified enzyme was shown to possess autophosphorylation activity, using Tyr 394 as a substrate (Ramer et al., 1991). This autophosphorylation activity is a common feature of *src*-related PTKs (Hunter, 1987 and Cantley et al., 1991) and most other PTKs such as the insulin receptor (Rosen et al., 1983) and the EGF receptor

(Bertics and Gill, 1985). Experimentally, the *in vitro* autophosphorylation activity of PTKs can be of great importance as it can be used to detect their presence. Together with co-immunoprecipitation techniques, it is possible to determine if certain surface proteins, such as adhesion molecules, are indeed associated with PTKs. These approaches have been used to investigate the signalling properties of CD4/CD8 and the TCR/CD3 complex, which were found to be associated with $p56^{lck}$ and $p59^{fyn}$, respectively.

The activity of $p56^{lck}$ can be negatively regulated by phosphorylation of Tyr 505, which is the major site of in vivo phosphorylation. This negative regulation was observed by Amrein and Sefton (1988) and others, who showed that the mutation of this Tyr 505 residue to phenylalanine confered to $p56^{lck}$ a constitutively active and oncogenic phenotype (Abraham et al., 1991b). The activation of $p56^{lck}$ by mutation of Tyr 505 results in the increased autophosphorylation of Tyr 394 and the increased T cell responsiveness to antigen stimulation (Abraham et al., 1991a). It was shown, however, that the constitutive activity of $p56^{lck}$ by Tyr 505 mutation requires an intact myristylation site and that it can be abrogated by mutating Tyr 394 autophosphorylation site (Abraham and Veillette, 1990).

Several lines of evidence suggest that the level of Tyr 505 phosphorylation, and hence the PTK activity of $p56^{lck}$, could be under the control of CD45, a phosphotyrosine-specific tyrosine phosphatase. Using CD45-negative T lymphoma cell lines, it was shown that the loss of CD45 could be correlated to an increased phosphorylation of $p56^{lck}$ at Tyr 505 (Ostergaard et al., 1989). Furthermore, the antibody-mediated co-clustering of CD45 with CD4 or CD8 inhibited the anti-CD4 or anti-CD8-mediated activation of $p56^{lck}$, suggesting that CD45 can regulate $p56^{lck}$ activity (Ostergaard and Trowbridge, 1990). The observations that $p56^{lck}$ and CD45 can be physically associated in human T lymphocytes (Schraven et al., 1991) and that Tyr 505-phosphorylated $p56^{lck}$ can serve as an in vitro substrate for CD45 (Tonks et al., 1990, Mustelin and Altman, 1990) strongly support the view that $p56^{lck}$ can be directly activated by CD45-mediated dephosphorylation of Tyr 505 (Mustelin et al., 1989 and Mustelin and Altman, 1990).

## 1.52 Adhesion molecules

Although TCR interaction with antigen/MHC is in itself sufficient to induce T cell activation, the physical contact between T lymphocytes and accessory cells is greatly facilitated by a variety of adhesion molecules expressed by interacting cells (reviewed by Springer, 1990). These adhesion molecules can be devided into "families", on the basis of

their common structures and their physical properties. The major families of adhesion molecules are as follows:

1) The immunoglobulin superfamily (e.g. CD2, LFA-3, CD4, CD8, ICAMs, NCAM and TCR/CD3), characterized by the presence of Ig-like domains or immunoglobulin homology units, a structure composed of a sequence of about 100 amino acids, stabilized by a centrally placed disulfide bridge and forming a series of anti-parallel β strands (Hunkapiller and Hood, 1986 and 1989).

2) The integrin family (e.g. LFA-1), includes heterodimeric glycoprotein adhesion molecules involved in cell-cell and cell-extracellular matrix interactions in the presence of divalent cations. A large number of integrins recognize a tripeptide (Arg-Gly-Asp or RGD) expressed by their counterreceptors (Hynes, 1987 and Ruoslahti and Pierschbacher, 1987). Synthetic peptides containing this RGD sequence can be used to block the interactions mediated by these integrins.

3) The selectin or LEC-CAM family (e.g. Mel-14), includes molecules characterized by the N-terminal domain homologous to a variety of calcium-dependent animal lectins and their capacity to mediate adhesion through glycosylated epitopes expressed by their ligands (Springer, 1990).

In the following sections, the most important adhesion molecules will be discussed, with a special emphasis on their structure, function and signal transduction properties. These adhesion molecules are also expressed by thymocytes and thymic stromal cells, suggesting that the intercellular interactions that they mediate may be important in T cell development. However, very little is known about their function in the thymus.

## 1.521 Thy-1

### 1.5211 Structure and function

Thy-1 was originally identified as a murine alloantigen expressed in two allelic forms (Thy-1.1 and Thy-1.2) on thymocytes, T lymphocytes, and nervous tissues (Reif and Allen, 1964). In the murine system, Thy-1 is also expressed by fibroblasts (McKenzie and Potter, 1979), Ia+ dendritic epidermal cells (Koning et al., 1987), bone marrow- and spleen-derived stromal cell lines (Pietrangeli et al., 1988) as well as by subpopulations of thymic stromal cells (Tucek and Boyd, 1990). Although no allelic forms were initially found for the human Thy-1 gene, Gatti et al. (1988) described a polymorphic Msp I site within the Thy-1 gene (on chromosome 11q22.3) distinguishing two alleles.

Thy-1 is a 25-30 kD glycoprotein encoded by genes related to the immunoglobulin supergene family (Williams and Gagnon, 1982). Amino-acid sequencing revealed that Thy-1 was composed of 111-112 amino acids, lacking a typical transmembrane domain. The cloning and sequencing of the Thy-1 gene revealed an additional segment of 20 hydrophobic amino acids (Seki et al., 1985). This carboxy-terminal hydrophobic stretch is cleaved from newly formed Thy-1 molecules to generate the mature protein, which is then attached to the membrane by a glycosyl-phosphatidylinositol (GPI) anchor (Tse et al., 1985, Homans et al, 1988, Low and Kincade, 1985). In fact, it has been shown recently that the inability of a cell to synthesize this glycosyl phosphatidylinositol anchor (i.e. to link glycan components to phosphatidylinositol prior to transfer to nescent peptides) abrogates the cell surface expression of Thy-1 molecules otherwise normally expressed by these cells (Stevens and Raetz, 1991, and Sugiyama et al., 1991).

**1.5212 Signal transduction through Thy-1.**

An increasing number of GPI-anchored proteins have been found on the lymphocyte membrane (Low, 1989). Most of these proteins, such as Qa-2 (Robinsion et al., 1989), Decay-accelerating factor or DAF (Davis et al., 1988), T cell-activating protein (TAP or Ly-6A.2; Rock et al., 1986) and 5'nucleotidase (CD73; Thompson et al., 1989), can function as

activation antigens in T cells; alone or in combination with other signals, they can promote T cell proliferation (Low and Saltiel, 1988). In the case of Thy-1, the full activation of mature T cells via the Thy-1 pathway seems dependent upon the co-expression of the TCR/CD3 complex (Schmitt-Verhulst et al., 1987 and Gunter at al., 1987). Using CTL clones and CTL variants, Guimezanes et al. (1988) found that Thy-1 specific mAbs could induce IFN-γ production by $TCR^+$ cytotoxic T cells, but not by $TCR^-$ CTL variants. Similarly, Thy-1-induced IL-2 production by $TCR^-$ T cell hybridomas can be restored by transfection of both TCR α and β genes, allowing the expression of a functional TCR/CD3 complex at the cell surface (Sussman et al., 1988b). These results indicate that that Thy-1-mediated signaling is not an alternative to, but is dependent on the TCR activation pathway. The recent observation that the TCR could be immunoprecipitated in association with Thy-1 and the protein tyrosine phosphatase CD45 supports the concept that the dependence of Thy-1 signalling on TCR coexpression might be due to their common interaction with CD45 (Volarevic et al., 1990).

The role of Thy-1 in T cell development is still a matter of speculation. Thy-1 expression on murine thymocytes reaches its maximum level around day 15 of gestation, i.e. immediately after the initial entry of pro-thymocytes into the thymus (Silver, 1989). Thy-1 surface expression decreases

significantly when mouse thymocytes migrates to the periphery and is totally absent from rat peripheral T cells (Silver, 1989). Like other members of the immunoglobulin supergene family expressed by T cells, namely CD2 and NCAM (neural cell adhesion molecule), Thy-1 can act as an adhesion molecule. The adhesion of mouse thymocytes to thymic stromal cells has recently been shown to be at least partly mediated by Thy-1 (He et al., 1991). Although no counterreceptor has been assigned to Thy-1, it was found that soluble Thy-1, isolated from mouse thymocytes, could interact with immobilized sulphated polysaccharides such as heparin and dextran sulfate (Parish et al., 1988). Interestingly, Thy-1 is also able to interact with matrix proteins, such as laminin and fibronectin, and tissue type plasminogen activator isolated from rat brain. Thy-1 bound the latter ligand through its lysine-containing domain (Liesi et al., 1990). Stimulation of fetal murine thymocytes (day 13) by either the CD3 or the Thy-1 pathway induced thymocyte proliferation as well as IL-2 and IL-4 secretion. The stimulation of day 15 fetal thymocytes by either pathway resulted in the expansion of a CD3/TCR$\gamma\delta^+$ thymocyte population (Tentori et al., 1988). It thus seems that either activation pathway can be used for the induction of fetal thymocytes, even at day 13, where no CD3/TCR expression can be detected by flow cytometry. However, the simultaneous stimulation of thymocytes from adult mice through both CD3 and Thy-1 pathways results in an increased and long-

lasting intracellular $Ca^{2+}$ rise, increased tyrosine phosphorylation of various intracellular substrates, DNA fragmentation and cell death (Nakashima et al., 1991). The same treatment also promoted cell death of thymocytes isolated from new born mice. The authors of these experiments suggest that Thy-1 might act in the thymus as an intensifier of CD3/TCR-mediated signals for the negative selection of autoreactive thymocytes.

## 1.522 CD2

### 1.5221 Structure and function

Previously known as T11, CD2 was originally identified as a T cell-specific sheep red blood cell (SRBC) receptor on human T cells (Lay et al., 1971). Monoclonal antibodies able to block this T cell/SRBC interaction recognize a non-polymorphic protein of 50kD (Howard et al., 1984). The molecular cloning of human (Sewell et al., 1986), rat (Williams et al., 1987) and mouse (Yagita et al., 1988) CD2 revealed a typical transmembrane protein having a 185-186 amino acid extracellular domain with several N-glycosylation sites, a transmembrane segment of 25 amino acids and a cytoplasmic domain of 116-126 residues. The gene encoding the CD2 molecule has been mapped to chromosome 1 in the human system and chromosome 3 in the murine system (Sewell et al.,

1987 and Clayton et al., 1988). CD2 shows limited regions of homology with members of the immunoglobulin supergene family (Williams et al., 1987).

CD2 can act as an adhesion molecule by interacting with LFA-3 (Lymphocyte function-associated antigen-3), its physiological ligand (Dustin et al., 1987a, Plunkett et al., 1987 and Selvaraj et al., 1987). LFA-3 is a 55-70 kD cell surface glycoprotein expressed by endothelial, epithelial and connective tissue cells as well as by a majority of blood cells, including erythrocytes (reviewed in Springer et al., 1987). In contrast to this wide distribution in human tissues, it was shown recently, using a soluble form of the murine CD2, that mouse LFA-3 is preferentially expressed by lymphoid cells. This suggests that the murine CD2/LFA-3 molecules are involved in lymphocyte/lymhocyte (T/T or T/B) rather than in lymphocyte/non-lymphoid cell interactions (Rutschmann and Karjalainen, 1991). The observation that CD2 is expressed by both murine T and B lymphocytes is in good agreement with this concept (Altevogt et al., 1989 and Yagita et al., 1989). A further molecular characterization of LFA-3 revealed a striking homology to its receptor CD2 (Seed, 1987). In addition, it was found that LFA-3 can be anchored to the plasma membrane by either a hydrophobic transmembrane domain or by a phosphatidyl inositol glycan moeity attached to the protein C-terminus (Dustin et al., 1987b).

## 1.5222 Signal transduction through CD2

The role of CD2 as an accessory T lymphocyte activation molecule has been demonstrated using different experimental approaches. First, it was observed that certain combinations of anti-CD2 monoclonal antibodies could induce IL-2-dependent T cell proliferation (Brottier et al., 1985), help for antibody responses (Meuer et al., 1984) as well as antigen-specific or -non specific cytolytic activity by cytotoxic T lymphocytes (CTLs) or natural killer (NK) cells, respectively (Siliciano et al., 1985). Anti-CD2 monoclonal antibodies can also activate CD3- or CD3+ thymocytes to express the IL-2R, but cannot induce proliferation (Fox et al., 1985). Second, it was shown that purified LFA-3, the receptor of CD2, can synergize with certain anti-CD2 mAbs to induce both human T lymphocyte proliferation (Tiefenthaler et al., 1987) and thymocyte activation (Denning et al., 1988b). Finally, transfection experiments demonstrated that the expression of LFA-3 by accessory cells provided a secondary signal facilitating both human T lymphocyte and thymocyte activation by either anti-CD3 mAbs or suboptimal doses of PHA (Bierer et al., 1988a), as well as the activation of a human T cell clone by its nominal antigen (Koyasu et al., 1990). Similarly, it was demonstrated that the reconstitution of CD2 expression by transfection of the human CD2 cDNA in CD3+CD2- T cell clones

increased the level of IL-2 responses triggered by TCR/CD3 stimulation (Makni et al., 1991).

Although a recent report indicated that the adhesion function of CD2 to LFA-3 is, by itself, sufficient to facilitate antigen recognition and TCR/CD3-mediated T lymphocyte activation (Moingeon et al., 1989), the observation that the CD2 intracytoplasmic domain is necessary to enhance CD3-mediated signals (Bierer et al., 1988b) confirmed that CD2 is involved the generation of an accessory activation signal. This concept was recently reinforced by the group of Reinherz (Chang et al., 1989 and Chang et al., 1990) who used a series of human CD2 cDNA deletion mutants to identify the regions and motifs of the 117 amino acid cytoplasmic tail of CD2 involved in signal transduction. While T lymphocyte activation via the TCR/CD3 complex is known to be independent of the surface expression of CD2 (Moingeon et al., 1988), whether or not TCR/CD3 expression is necessary for CD2-mediated T cell activation is still a matter of debate. Earlier reports regarding the ability of mitogenic anti-CD2 mAbs to activate $CD3^-$ thymocytes (Fox et al., 1985), natural killer cells (O'Flynn et al., 1986) or CD3- Jurkat mutants (Moretta et al., 1987) indicated that CD2 signalling does not always require the expression of a functional TCR/CD3 complex. More recently, however, evidence has been accumulated to indicate that TCR/CD3 expression is usually necessary for CD2-mediated

activation. For exemple, the anti-CD3-induced TCR/CD3 modulation or internalization in peripheral blood T cells inhibits anti-CD2-mediated T cell activation (Breitmeyer et al., 1987). Similarly, the transfection of a TCR ß chain-negative (and, hence, $CD3^-$) Jurkat mutant with a TCR ß chain cDNA has been shown to restore CD3/TCR expression as well as the activation via the CD2 pathway (Bockenstedt et al., 1988, and Alcover et al., 1988). Most recently, Ohno et al. (1991) reported that human and mouse T cells can be activated via the CD2 pathway in absence of a functional TCR/CD3 complex, only if CD2 is expressed at high density at the cell surface. This report may well have provided the solution to the controversy regarding the requirement of TCR/CD3 expression in T cell activation through the CD2 pathway.

The true nature of the relation between CD2 and the TCR/CD3 complex might well be their common association with the $p56^{lck}$ protein tyrosine kinase, which can be activated by either pathway (Danielian et al., 1989 and 1991). The activation of PKC which has been shown to result from CD2 stimulation (Friedrich et al., 1989) might well be, as in the case with TCR/CD3, a result of $p56^{lck}$ activation. The possibility that CD2 might be functionally associated with $p56^{lck}$ was recently strengthened by several reports showing that the CD2-mediated T cell stimulation was in fact under the control of the protein tyrosine phosphatase CD45 (Samelson et

al., 1990b and Koretzky et al., 1991), which was found to be physically associated to CD2 (Schraven et al., 1990), and postulated to be involved in $p56^{lck}$ activation. This set of observation is important and of particular relevance to the present work, because it demonstrates that, similarly to the T cell antigen recognition system, cell adhesion molecules can use a protein tyrosine kinase pathway for signal transduction.

## 1.523 LFA-1 (Leukocyte function-associated molecule 1)

### 1.5231 Structure and function

LFA-1 was originally identified as a mouse lymphocyte surface antigen participating in T lymphocyte-mediated killing (Davignon et al., 1981). This was later shown in the human system (Sanchez-Madrid et al., 1982). LFA-1 is composed of two subunits, $\alpha$ (180 kD) and $\beta$ (95 kD). It is expressed on all leukocytes and their progenitors (Kurzinger et al., 1981 and Arnaout, 1990). Its function is necessary to many immunological processes involving cell-cell contact, such as lymphocyte adhesion to endothelial cells (Haskard et al., 1986, Dustin and Springer, 1988, Hamann et al., 1988 and Smith et al., 1989), conjugate formation between CTLs, NK cells and lymphokine-activated killer (LAK) cells on the one hand, and their respective targets on the other hand (Davignon et al., 1981, Krensky et al., 1983, Shaw et al., 1986 and Nakamura et

al., 1990), as well as the mixed lymphocyte reaction (MLR; Bagnasco et al., 1990). LFA-1 also participates in T cell-T cell and T cell-B cell interactions (Boyd et al., 1988, Mazerolles et al., 1988, and Sanders and Vitetta, 1991). The observation that LFA-1 is involved in the adhesion of immature ($CD3^{lo}$) thymocytes to thymic epithelial cells in both the mouse (Lepesant et al., 1990) and the human (Nonoyama et al., 1989 and Singer et al., 1990) systems lead to the hypothesis that LFA-1-dependent interactions between thymocytes and thymic stromal cells might be important in T cell development. This was recently confirmed using a fetal thymic organ culture (FTOC) system, in which anti-LFA-1 mAbs were added. This treatment resulted in an impaired production of CD4+CD8+ thymocytes from more immature populations, which were not themselves affected by the treatment (Fine and Kruisbeek, 1991).

LFA-1 is a member of the Integrin family of adhesion molecules. The integrins are a group of heterodimeric glycoproteins consisting of noncovalently associated α and β subunits, which were originally divided into three groups (reviewed by Buck and Horwitz, 1987, Ruoslahti and Pierschbacher, 1987, Hynes, 1987, Albelda and Buck, 1990, ). Each group is defined by a common β chain. Hence, the function of each individual member of a group is defined by its unique α chain. More recent work has shown that there are at least

six different β subunits, and that certain α subunits can combine with more than one β subunit (Cheresh et al., 1989, Vogel et al., 1990 and Krissansen et al., 1990). LFA-1 (also called CD11a/CD18 or $\alpha_L\beta_2$) is a member of the $\beta_2$ subfamily, along with MAC-1 (CD11b/CD18 or $\alpha_M\beta_2$) and p150,95 (CD11c/CD18 or $\alpha_X\beta_2$). In the human system, the genes coding for α chains of all three members of the $\beta_2$ family have been located on chromosome 16, whereas the gene encoding the common β chain is located on chromosome 21 (Corbi et al., 1988 and Solomon et al., 1988). The structure of LFA-1 contains functional domains which are common to all integrins. The β chain, homologous to the fibronectin receptor, contains 4 repeats of a 40 amino-acid cystein-rich motif, producing extensive intrachain disulfide bonding (Hynes, 1987 and Law et al., 1987). The large extracellular domain of the LFA-1 α chain contains seven 60 amino acid internal repeats, of which the three closest to the extracellular COOH-terminal contain divalent cation binding sites (Larson et al., 1989). This cation-binding might be an important structural feature, since LFA-1-mediated interactions require the presence of $Mg^{++}$ and $Ca^{++}$ (Marlin and Springer, 1987). Several members of the integrin family of adhesion molecules interact with components of the extracellular matrix, mainly through the "RGD" (Arg-Gly-Asp) tripeptide, which is the integrin binding site (Ruoslahti and Pierschbacher, 1987). LFA-1-dependent adhesion, however, has

been shown to be "RGD"-independent (Marlin and Springer, 1987 and Wawryk et al., 1989).

## 1.5232 LFA-1 ligands

LFA-1 can interact with at least three different ligands. First, the human ICAM-1 (Intercellular adhesion molecule-1, CD54) was identified as an LFA-1 counterreceptor by the ability of an anti-ICAM-1 mAb to block the LFA-1-dependent homotypic adhesion of stimulated T and B lymphocytes (Rothlein et al., 1986). Direct evidence that ICAM-1 could act as an LFA-1 ligand was provided by the observation that purified ICAM-1, incorporated into artificial lipid membranes (Marlin and Springer, 1987), or coated onto a solid matrix (Makgoba et al., 1988) could interact with B, T and myeloid cells in an LFA-1-dependent fashion. ICAM-1 is expressed on a variety of non-hematopoietic cells such as vascular endothelial cells, epithelial cells from the thymus and other origins as well as fibroblasts. It is also expressed by hematopoietic cells such as tissue macrophages, mitogen-stimulated T cells and dendritic cells in the germinal centers of lymph nodes and Peyer's patches (Dustin et al., 1986). The level of ICAM-1 expression on the surface of fibroblasts and endothelial cells can be up-regulated (2.5 to 40 fold) by treatment with inflammatory mediators such as IL-1, IFN-$\gamma$, lipopolysaccharides (LPS) or tumor necrosis factor (TNF;

Dustin et al., 1986, Pober et al., 1986, and Dustin and Springer, 1988). ICAM-1 is a 90 kD glycoprotein (Rothlein et al., 1986). Its amino acid sequence revealed an integral membrane protein with an extracellular portion of 453 amino acid residues containing five immunoglobulin-like domains (Staunton et al., 1988) highly homologous to those found on two neural adhesion molecules, namely NCAM (neural cell adhesion molecule; Simmons et al., 1988) and MAG (myelin-associated glycoprotein). The $NH_2$-terminal immunoglobulin-like domain of ICAM-1 has been shown to contain the LFA-1 binding site, as well as a distinct but overlapping binding site for a major group of rhinoviruses (Staunton et al., 1990). Interestingly, ICAM-1 is also able to act as a counter-receptor for another integrin, namely Mac-1 ($\alpha_M\beta_2$; Diamond et al., 1990). The binding site for Mac-1 on ICAM-1, distinct form the one for LFA-1, has been located on the third $NH_2$-terminal immunoglobulin-like domain of ICAM-1 (Diamond et al., 1991). The murine homologue of ICAM-1, called MALA-2, has been identified by Pietro et al.(1989) and its function, cellular distribution and molecular properties are indistinguishable from those of its human counterpart.

In many systems, anti-ICAM-1 antibodies were found to be unable to totally block LFA-1-dependent adhesion, and it was therefore concluded that LFA-1 could interact with more than one ligand (Rothlein et al., 1986). This was supported by the

identification of an LFA-1-dependent but ICAM-1-independent adhesion pathway to endothelial cells (Dustin and Springer, 1988) and by the observation that target cell adhesion with and lysis by CTLs could be LFA-1-dependent but ICAM-1-independent (Makgoba et al., 1988). A second ligand for LFA-1, namely ICAM-2, was identified by the functional cloning of a cDNA able to confer adhesiveness of transfected COS cells to purified LFA-1 in presence of anti-ICAM-1 mAb (Staunton et al., 1989). The deduced amino acid sequence revealed an integral membrane protein with two immunoglobulin-like domains closely related to the two N-terminal immunoglobulin-like domains of ICAM-1. Recently, mAbs raised against ICAM-2 revealed a 55-60 kD glycoprotein (Nortamo et al., 1991 and de Fougerolles et al., 1991) expressed by lymphoblastoid B cells, resting lymphocytes, vascular endothelial cells and mitogen-stimulated blood mononuclear cells. In contrast to ICAM-1, ICAM-2 expression cannot be induced by inflammatory mediators.

While the LFA-1-dependent homotypic aggregation of a transformed B cell line could be totally blocked by anti-ICAM-1 plus anti-ICAM-2 mAbs, the LFA-1-dependent aggregation of a T cell lymphoma cell line could not be inhibited by the same treatment. This was interpreted as evidence for the presence of a third LFA-1 ligand (de Fougerolles et al., 1991). This was indeed verified recently by the identification of a monoclonal antibody that could block completely the ICAM-1 and

ICAM-2-independent adhesion of T and B cell lines to purified LFA-1. The antigen defined by this antibody was called ICAM-3 and is a highly glycosylated protein of 124 kD. ICAM-3 is expressed on all leukocytes and totally absent from endothelial cells (de Fougerolles and Springer, 1992). On the basis of adhesion inhibition studies, it was concluded that ICAM-3 was the most active LFA-1 ligand expressed by resting lymphocytes.

**1.5233 Regulation of LFA-1-dependent adhesion.**

The mechanisms by which LFA-1-dependent interactions are regulated are multiple and complex. One way to increase LFA-1-dependent adhesion is the upregulation of either LFA-1 or its ligand(s) surface expression. For example, treating B cells with anti-CD40 mAbs (CD40 is a putative cytokine receptor; Clark, 1990) induces ICAM-1 expression (Barrett et al., 1991), while a treatment of B cells with anti-IgM antibodies, which mimick the engagement of surface Ig receptor or antigen stimulation, induces the expression of both ICAM-1 and LFA-1 (Dang and Rock, 1991). Similarly, the treatment of neutrophils with anti-CD15 mAbs, recognizing a glycosylated epitope of neutrophil adhesion molecules, induces LFA-1 expression and neutrophil adhesion to endothelial cells (Forsyth et al., 1989).

In contrast to B cell Ig receptor engagement, TCR stimulation results in a rapid and transient induction of LFA-1 adhesive activity, but not in the upregulation of LFA-1 expression (Dustin and Springer, 1989 and Moingeon et al., 1991). The same increase in LFA-1 avidity can be achieved by T cell stimulation through CD2, in which case the activation of LFA-1 was more sustained (van Kooyk et al., 1989). Similarly, T cell stimulation through CD44, an adhesion molecule previously known as Pgp-1 or Hermes-1 (St. John et al., 1990), also increases LFA-1 adhesiveness (Koopman et al., 1990). The TCR-driven conversion of LFA-1 to a high avidity state is protein kinase C (PKC)-dependent (Dustin and Springer, 1989 and van Kooyk et al., 1989) and can therefore be directly induced by phorbol esters (Rothlein et al., 1986). Interestingly, the TCR-driven induction of LFA-1 can be inhibited by several CD4 ligands, such as anti-CD4 mAbs, the gp160 component of HIV and synthetic peptides derived from the HLA-DR binding sequence of CD4 (Mazerolles et al., 1991). While the mechanisms of LFA-1 conversion from low to high avidity states are still nebulous, recent reports indicated that the intact intracellular domain of the β subunit of LFA-1 was required (Hibbs et al., 1991). Furthermore, it was found that the LFA-1 molecule at the surface of TCR/CD3-activated T cells express a unique $Ca^{++}$-dependent activation epitope, defined by the NKI-L16 anti-LFA-1 mAb, which is absent from $LFA\text{-}1^{+}$ resting T cells (van Kooyk et al., 1991). The

expression of this epitope can be correlated to the high avidity state of LFA-1 and suggests that LFA-1 induction requires some conformational modification of pre-existing LFA-1 molecules.

## 1.5234 Signal transduction through LFA-1.

It is now generally accepted that LFA-1 engagement can result in the delivery of co-stimulatory signals, at least in T cells. Indeed, it was shown that anti-LFA-1 mAbs, used together with submitogenic doses of anti-CD3 mAb or phorbol myristate acetate (PMA), could induce IL-2-dependent T cell proliferation (Carrera et al., 1988). Similarly, LFA-1 ligands ICAM-1 and ICAM-2 were found to have the same co-stimulatory effect on anti-CD3-triggered T cell proliferation (van Noesel et al., 1988, Van Seventer et al., 1990 and Damle et al., 1992). LFA-1 cross-linking using anti-α chain mAbs results in phosphoinositide hydrolysis and a rise in intracellular calcium, while cross-linking with an anti-β chain mAb has no effect (Pardi et al., 1989 and Wacholtz et al., 1989). Furthermore, it was recently shown that LFA-1 cross-linking at the surface of an activated human T cell clone (HuT 78/2) was sufficient to induce a cytoskeletal reorganisation resulting in the development of dendritic processes (Kelleher et al., 1990).

Finally, it is noteworthy that LFA-1 signals have also been shown to synergize with CD2-delivered signals, resulting in an increased T cell proliferation (Cerdan et al., 1989). This CD2/LFA-1 cooperation in signal delivery has recently been shown to be active in T cell activation by anti-CD3 mAbs in presence of autologous monocytes, suggesting that both LFA-1 and CD2 can be involved in the generation of co-stimulatory signals during T cell activation by antigen-presenting cells (Van Seventer et al., 1991).

## 1.524 NCAM (Neural cell adhesion molecule).

### 1.5241 Structure and function

NCAM is a member of the immunoglobulin supergene family and is composed of five immunoglobulin-like domains and two fibronectin type III segments (Edelman, 1986 and Cunningham et al., 1987). It is related to other neural cell adhesion molecules such as MAG (myelin-associated glycoprotein) and the J1 glycoprotein, with both of which NCAM share a common carbohydrate moeity recognized by mAbs L2 and HNK-1 (Kruse et al., 1984, Kruse et al., 1985 and Poltorak et al., 1987). NCAM can be expressed as several isoforms (at least eight are known in the mouse; Santoni et al., 1989) generated by alternative splicing (Barthels et al., 1988 and Andersson et al., 1990) and polyadenylation (Barbas et al., 1988) of a single mRNA.

Transmembrane isoforms of 140 and 180 kD and a 120 kD phosphatidylinositol-glycan anchored isoform have been identified in neural and muscle tissues (Cunningham et al., 1987). NCAM-mediated adhesion is probably homotypic, as suggested by Mege et al. (1988), who showed that liposomes bearing purified NCAM could bind to a mouse sarcoma cell line only if it had been transfected with the NCAM cDNA.

Lymphocytes, their precursors and, in fact, all cells of hemopoietic tissues were initially believed to lack NCAM, but it has finally been shown that stromal cells from mouse bone marrow and spleen synthesize this adhesion molecule (Thomas et al., 1988). Recently, the expression of NCAM by subpopulations of fetal thymocytes ($CD4^+CD8^+$) has been reported (Brunet et al., 1989). The expression of the adhesion molecule was maximum at birth and declined progressively until day 21 after birth, where it became negative. At this point, a role of NCAM in early T cell development can only be postulated.

**1.525 Mel-14**

Mel-14 is a lymph node homing receptor expressed by mature T cells, as well as thymocytes having a mature phenotype (Reichert et al., 1986, reviewed by Gallatin et al., 1986, Yednock and Rosen, 1989, and Springer, 1990). It is a member of the selectin or LEC-CAM family of adhesion molecules, which

includes GMP-140 (a platelet adhesion molecule) and ELAM-1 (an inducible adhesion molecule expressed by endothelial cells). The Mel-14 adhesion molecule is a 90 kD glycoprotein (Gallatin et al., 1983) which interacts, in a sugar-inhibitable manner, with peripheral lymph node high endothelial venules (HEV; Geoffroy and Rosen, 1989). The key feature of the Mel-14 molecule was revealed by Lasky et al. (1989), who showed the presence of a N-terminal domain highly homologous to $Ca^{++}$-dependent animal lectins. This domain is directly responsible for cell-cell adhesion, since an antibody which binds to the lectin portion of Mel-14 completely blocks adhesion to HEVs (Bowen et al., 1990). Therefore, all selectin-mediated adhesions depend on the presence of $Ca^{++}$ for their interaction with carbohydrate ligands.

## 1.6 Hypothesis and objectives.

The informations presented in section 1.5, and particularly the physical properties governing the adhesions mediated by each of these systems, can now be compared with those governing the interaction of $CD4^+CD8^+$ thymocytes (and Ti-6 lymphoma cells) with E-5 (table 1). This allows us to determine whether one of these adhesion molecules might be involved in E-5/thymocyte adhesion.

This comparison led us to hypothesize that the interaction of $CD4^+CD8^+$ thymocytes with E-5 cells is mediated by an epithelial adhesion molecule different of those which have been described thus far in the literature. Therefore, the main objective of this work was to identify and purify this putative E-5 adhesion molecule, and to show its capacity to mediate the adhesion of $CD4^+CD8^+$ thymocytes *in vitro*. Finally, since adhering thymocytes did not appear to be affected by their interaction with E-5 cells, it was important to verify the ability of this $CD4^+CD8^+$ thymocyte adhesion to trigger the activation of the E-5 epithelial cells (see section 1.4) and to identify the signal transduction mechanism induced by adhesion through the E-5 adhesion molecule.

| | Temp. | $CD4^+CD8^+$ | $Ca^{++}/Mg^{++}$ | Glyco. | Homo. |
|---|---|---|---|---|---|
| E-5 | 4°C/37°C | YES | NO | NO | NO |
| Integrins | 37°C | NO | YES | NO | NO |
| Ig. | 4°C/37°C | NO | NO | NO | NO |
| Selectins | 37°C | NO | YES | YES | NO |
| NCAM | 37°C | YES | NO | NO | YES |

Table 1: Comparison of the physical properties governing the adhesion mediated by E-5 and by the members of the major families of adhesion molecules (integrins, immunoglobulines [Ig.], selectins and NCAM). The factors considered in this comparison are respectively: temperatures (temp.) at which adhesion is possible; selective binding to $CD4^+CD8^+$ thymocytes; dependence of the interaction on the presence of divalent cations ($Ca^{++}/Mg^{++}$); interaction with a glycosylated ligand (Glyco) and homotypic (homo) interactions.

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## 2.2 Introduction

The identification and purification of the putative E-5 adhesion complex first requires a functional adhesion assay capable to measure the thymocyte adhesion activity of any E-5 membrane protein, once it has been solubilized and removed from its natural membrane environment. The solubilized E-5 adhesion molecule must therefore retain its original adhesion function and be immobilized in a "membrane-like" configuration. This was elegantly demonstrated in a previous investigation of this system (Potworowski et al., 1986a), where it was shown that a glycoprotein fraction from E-5 cells could block thymocyte adhesion to E-5 cells more efficiently if the E-5 glycoproteins were incorporated into liposome membranes.

Functional E-5 surface glycoproteins could therefore be incorporated into proteoliposome membranes and adsorbed on either glass coverslips (forming supported planar membranes) or cell-sized silica beads (forming structures called pseudocytes), which would provide a solid support for the artificial membranes. The first approach was developed by Watts for the study of the requirements of antigen presentation to T cells by purified and immobilized class II MHC products (Watts et al., 1984, 1985, Watts and McConnell, 1986 and Watts, 1988). Similarly, pseudocytes were developed

by Mescher's group for the study of CTL activation by purified and immobilized class I MHC antigens (Goldstein and Mescher, 1986, 1988 and Kane et al., 1988). Both systems provide a lipidic membrane-like environment to the isolated proteins and preserve their function. Although supported planar membranes have been used to demonstrate that purified ICAM-1 was a ligand for LFA-1 (Marlin and Springer, 1987), these systems are rarely used for the identification or characterization of cell-cell adhesion molecules. Furthermore, the fact that only 50% (or less) of the immobilized proteins are actually exposed at the surface of the pseudocytes (Goldstein and Mescher, 1986) or planar membranes (Watts et al., 1984) makes these approaches less attractive when the amount of protein is limited.

An alternative approach is the direct adsorption of membrane proteins to plastic in a low concentration detergent-containing buffer. Such strategy was used by Springer's group for the identification of LFA-1 and Mac-1 ligands (Staunton et al., 1989, Diamond et al., 1990 and de Fougerolles and Springer, 1992). The presence of low concentrations of detergent was shown to allow the binding of membrane proteins such as MHC antigens, while inhibiting the simultaneous binding of soluble proteins such as immunoglobulins (Kane et al., 1989a). These contrasting properties led the authors to suggest that membrane proteins could, in these conditions,

bind the plastic through their exposed hydrophobic regions and thus be "oriented" on the plastic surface. This is supported by the observation that class I MHC proteins, immobilized onto PVC plates using this method, can activate CTLs (Kane et al., 1989a and 1989b) through a CD8-dependent mechanism (O'Rourke et al., 1990).

The following series of experiments represent an attempt to adapt this strategy to the E-5 system. It is therefore vital to verify that an E-5 glycoprotein extract immobilized in this fashion can mediate thymocyte adhesion and that this assay can allow the accurate detection of thymocyte binding activity if the original extract is fractionated.

## 2.3 Materials and Methods

### 2.31 Cell lines

The E-5 cell line was derived from a primary culture of thymic stromal cells from 4-5 weeks old C57Bl/6 mice (Potworowski et al., 1986b). The cells were transformed using the chemical carcinogen n-methyl-n'-nitro-n-nitrosoguanidine (MNNG; Steele et al., 1977). The phenotype of E-5 cells is characteristic of thymic medullary epithelial cells (Potworowski et al., 1986b), since they express cytokeratin and react with medullary epithelial cell-specific mAbs ERTR5

and ERTR6 (Van Vliet et al., 1984). E-5 cells were grown at 37°C, 5% $CO_2$, in a 1:1 mixture of medium 199 supplemented with Hank's salts and MEM (Eagle) supplemented with Earle's salts to which "Nu Serum" (Collaborative Research, Lexington, MA) was added to a final concentration of 10 %.

RDM4 cells, a gift from Dr D. Oth, derived from an AKR thymic lymphoma, were cultured at 37°C, 5% $CO_2$, in RPMI 1640 containing 10% FCS.

Thymocytes were prepared fresh by mincing thymuses from C57BL/Ka mice in cold Hank's balanced salt solution (HBSS; Gibco, Grand Island, NY). The cell suspensions were passed through a fine nylon wool column, washed by centrifugation (250g for 10 min) and resuspended in HBSS containing 2% FCS.

## 2.32 Isolation of E-5 membrane glycoproteins (E-5gp).

E-5 cell membranes were obtained by nitrogen decompression using a pressure homogenizer (Parr). This apparatus, first described by Hunter (1961) allows the use of isotonic conditions for membrane disruption. In the present case, the STEM buffer (250 mM sucrose, 10 mM Tris, 1 mM EGTA and 1 mM $MgCl_2$, pH 7.4; described by Islam et al., 1983) was used. E-5 cells were therefore suspended in STEM at a concentration of $10^9$/100 ml and pressurized in the nitrogen bomb at 625 PSI for

15 minutes. After pressurization, the gradual decompression of the cells in the STEM buffer allows the selective disruption of the plasma membrane, without damaging the nucleus and other organelles. The enriched E-5 plasma membranes were isolated by differential centrifugation (Islam et al., 1983) and resuspended in TBS (50 mM Tris, 150 mM NaCl, pH 8.0) containing 1 mM PMSF. The protein content of the E-5 membrane suspension was determined using the BCA reagent (Pierce, Rockford, IL) and the membranes were solubilized using 0.25% laurylmaltoside (Rosevear et al., 1980; detergent to protein ratio of 2:1) in TBS.

E-5 membrane glycoproteins were isolated by affinity chromatography using a lentil lectin-Sepharose column (Pharmacia, Uppsala, Sweeden) equilibrated with TBS containing 0.25% lauryl maltoside. Unbound proteins were washed using TBS and E-5 glycoproteins were eluted with 0.5 M glucose in TBS (Potworowski et al., 1986a). The E-5gp fraction was concentrated by ultrafiltration using a CX-10 filter (Millipore) and dialysed against PBS containing 0.01% sodium deoxycholate (DOC).

## 2.33 Solid-phase adhesion assay.

The methods used for this assay have been adapted from Kane et al., 1989. Briefly, indicated amounts of E-5gp (in

PBS/0.01% DOC) were plated in a volume of 50 $\mu$l into microtiter wells (96 wells Falcon microtest III PVC plates, Becton Dickinson, Oxnard, CA). The plates were incubated at 25°C for 90 minutes and at 4°C overnight. Unbound proteins were removed by three consecutive washes with 250 $\mu$l PBS and the wells were blocked for 90 minutes at 25°C using PBS containing 5% BSA. After removal of the blocking solution, 5 x $10^5$ chromium-labelled thymocytes were added to each well in a volume of 55 $\mu$l $Ca^{2+}$ and $Mg^{2+}$-free PBS containing 2% BSA, and the plates were incubated for 90 minutes at 4°C, which correspond to the conditions previously used to characterize the adhesion of thymocytes to E-5 cells (Hugo et al., 1988, Hugo and Potworowski, 1989 and 1990). Non-adhered thymocytes were removed by five consecutive washes with 250 $\mu$l cold PBS. Adhered thymocytes were lysed with 100 $\mu$l 2% SDS, and the released chromium counted using a Gamma 7000 counter (Beckman, Palo Alto, CA). In these experiments, the adhesion of thymocytes to immobilized RDM4 membrane glycoproteins was used as a negative control. The level of adhesion, expressed as % adhesion, was calculated as follows:

$$\% = \frac{\text{(cpm bound to E-5gp/RDM4) - (cpm bound to plastic)}}{\text{(total cpm for } 5\text{x}10^5 \text{ thymocytes)}} \times 100$$

## 2.34 Fractionation of E-5gp by ion exchange chromatography.

A preliminary fractionation of the E-5gp fraction was performed by ion exchange chromatography using a DEAE-5PW column coupled to a HPLC system (Waters). Briefly, 120 µg of E-5gp in 1.2 ml 50 mM Tris, pH 8.0 were injected into the column, and the non-retained material was collected by washing the column with 50 mM Tris, pH 8.0. The retained glycoproteins were eluted using a linear gradient of NaCl (0 to 1 M NaCl in 50 mM Tris, pH 8.0). Each collected peak was concentrated by ultrafiltration and dialysed against TBS containing 0.01% DOC. For the measurement of the thymocyte adhesion activity, 2 µg of each fraction were immobilized into PVC plates.

## 2.4 Results and discussion

### 2.41 Direct adhesion of thymocytes to E-5gp

The E-5gp fraction, known to inhibit the adhesion of thymocytes to E-5 cells in previous experiments (Potworowski et al., 1986a) was expected to contain the active E-5 adhesion molecule. This fraction was therefore used for the development of our adhesion assay. The immobilization of E-5gp into PVC plates, in the presence of low concentrations of detergent (0.01% DOC), led to a reproducible binding of chromium-labelled thymocytes. This binding was dose-dependent and

seemed to plateau at 2 μg of E-5gp (figure 1). However, when an irrelevant lentil lectin-binding glycoprotein extract (RDM4 lymphoma cells) was used at the same concentrations, no thymocyte binding was observed.

The fact that the thymocyte adhesion level reaches a maximum between 1 and 2 μg of E-5gp per well may reflect the possibility that the protein binding sites are saturated at this protein concentration. A similar observation was made by Kane et al. (1989), who showed a saturation of the binding of purified H-2K$^k$, I-A$^k$, and other MHC antigens to the same PVC plates, at a concentration of 0.3 μg per well. The apparent discrepancy in the saturation concentrations observed in both systems (1-2 μg/well vs 0.3 μg/well) might be explained by the presence of high molecular weight glycoproteins in the E-5gp extract.

## 2.42 The E-5 adhesion molecule is composed of at least two subunits

To further demonstrate the specificity of thymocyte adhesion to E-5gp and to determine whether or not this assay could be used to monitor the presence and the enrichment of the adhesion molecule throughout its purification, the E-5gp fraction was fractionated by ion exchange chromatography on a DEAE-5PW column. The elution profile shown in figure 2

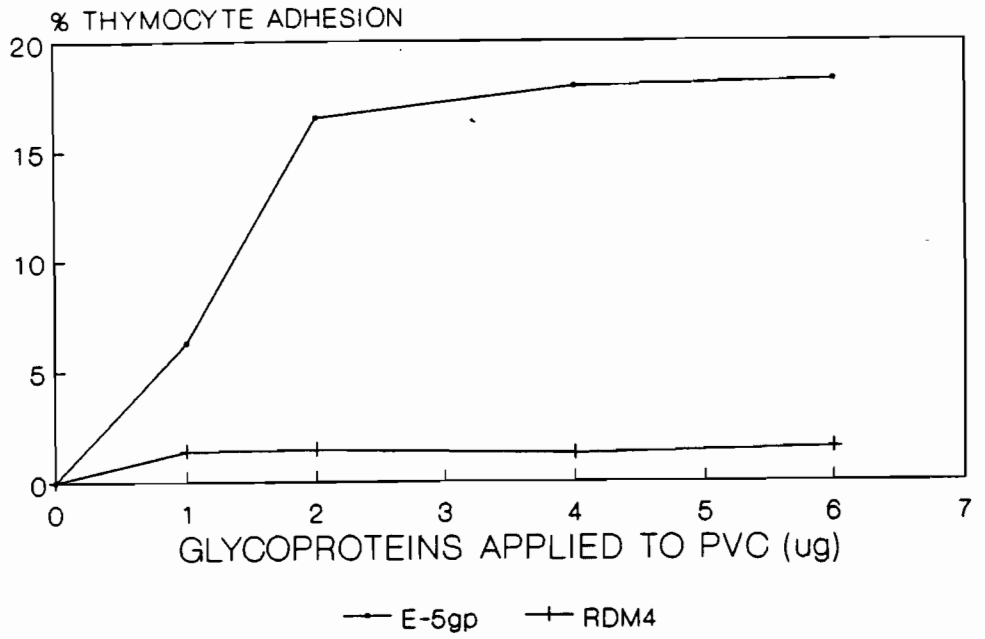


**Figure 1:** <u>Detection of thymocyte adhesion activity in the E-5gp extract.</u> **Chromium-labeled thymocytes were incubated with increasing amounts of PVC-immobilized glycoprotein fractions of either E-5 cells or RDM4 cells. A dose-dependent adhesion of thymocytes to E-5gp, but not to RDM4, was observed. A maximal level of thymocyte adhesion is reached at 2 μg E-5gp per well.**

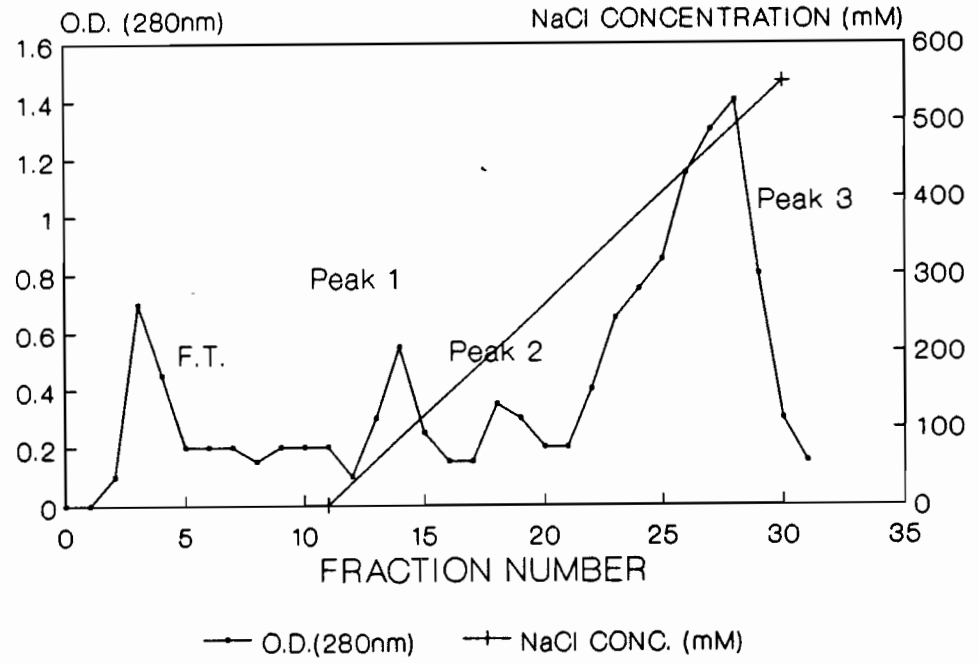


**Figure 2:** Fractionation of E-5gp by ion exchange chromatography. **E-5gp was subected to cation exchange chromatography using a DEAE 5PW column. The elution profile (O.D. 280 nm, left y axis) shows the presence of a non-retained (F.T. or fall through) fraction as well as three "retained" fractions (peak 1, peak 2, and peak 3) which were eluted using a linear NaCl gradient (right y axis). Each collected fraction (x axis) had a volume of 1.5 ml.**

indicates that E-5gp could be fractionated into at least 4 distinct fractions using this procedure, the bulk of glycoproteins (more than 80%) being eluted at 300-500 mM NaCl.

To determine if the thymocyte adhesion activity could be detected in any of these fractions, 2 μg of each fraction were immobilized into PVC plates and tested for thymocyte binding activity. As expected, the original E-5gp fraction was found to be active (figure 3). However, neither the non-retained (F.T.) fraction nor any of the retained fractions (peaks 1, 2, and 3, individually immobilized) were found to bind thymocytes. Similarly, when all retained fractions were plated together (in a final concentration of 2 μg/well), no adhesion activity could be detected. However, when the F.T. fraction was immobilized in association with all three retained fractions, an adhesion activity similar to the original E-5gp fraction was observed. These results show that the F.T. fraction is required, but not sufficient, for thymocyte adhesion.

To determine which "retained" fraction is conjointly necessary for thymocyte binding, the F.T. fraction was plated successively with each individual "retained" fraction. When the peak 2 fraction was added to F.T., we observed a high level of thymocyte binding, whereas the addition of either peak 1 or peak 3 to F.T. resulted in no adhesion. These

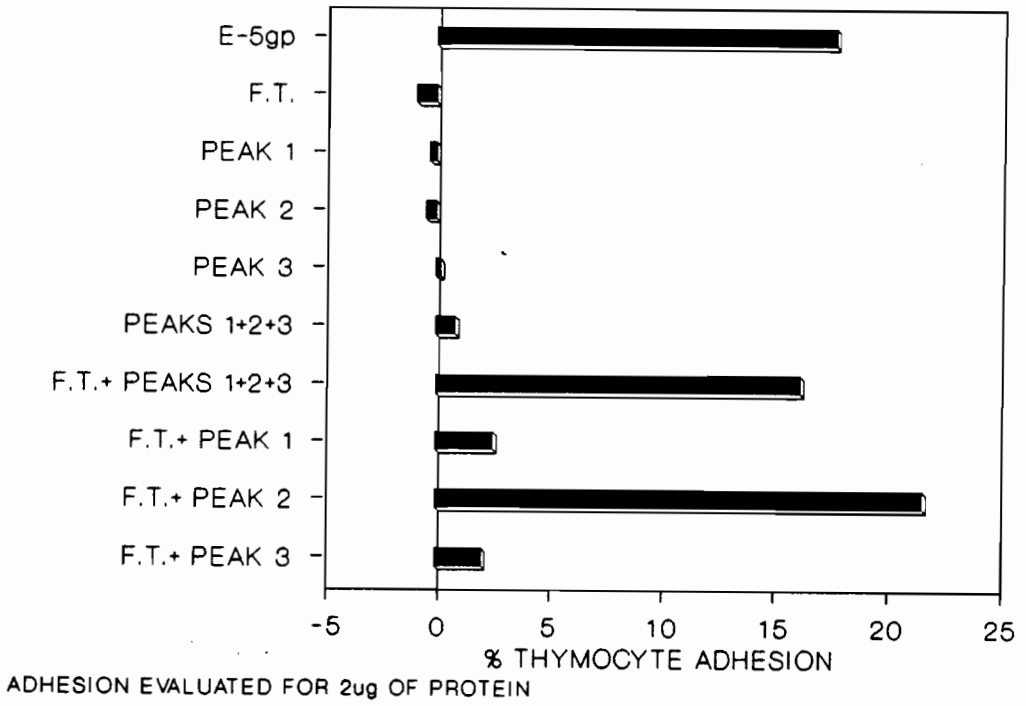


**Figure 3:** <u>Measure of the thymocyte adhesion activity of fractionated E-5gp.</u> In vitro adhesion of chromium-labeled thymocytes to PVC-immobilized E-5gp and combinations of F.T., peak1, peak 2 and peak 3 fractions. The total protein concentration was 2 μg of protein per well in each case. The adhesion activity present in E-5gp was disrupted during the fractionation of the extract, but could be reconstituted by co-immobilizing proteins of all four fractions or proteins of the F.T. and peak 2 fractions.

results strongly suggest that the E-5 adhesion molecule is composed of at least two subunits which bear a different electric charge at pH 8.0, allowing their efficient separation by ion exchange chromatography. Furthermore, we can conclude that this "molecular adhesion assay" can be used to identify the E-5 adhesion molecule, since it can detect adhesion activity in the crude E-5gp extract (figure 1) and in fractionated E-5gp (figure 3). The fact that the level of thymocyte adhesion is higher with the F.T./Peak 2 combination than in the starting E-5gp extract (21.7% vs 18%) may reflect a certain enrichment of the E-5 adhesion molecule. If that is indeed the case, one might expect that the purified adhesion molecule will give a maximum thymocyte adhesion level inferior to 80%, since it was observed that E-5 cells can interact almost exclusively with $CD4^+CD8^+$ thymocytes (Hugo and Potworowski, 1990), a population which represents 80-85% of adult thymocytes (reviewed by Adkins et al., 1987 and Folkes and Pardoll, 1989).

Although these observations suggest that the E-5 adhesion molecule could be identified after further fractionations of the F.T and peak 2 fractions by HPLC chromatography, the large number of different glycoproteins observed in these two fractions prevented us from using this approach. A simpler and more classical strategy has therefore been used. Rat monoclonal antibodies have been raised against the entire E-

5gp fraction and screened for their ability to block thymocyte adhesion to E-5 cells. Such antibodies have been used to identify the E-5 adhesion molecule, and had the additional advantage to allow the identification of associated structures which are not involved in thymocyte adhesion.

**2.43 Post script.**

At the time that these experiments were conducted, there was no specific reagent to the E-5 adhesion molecule. However, the development of monoclonal antibodies L-43 (chapter 3) and C3C12 (chapter 4), capable of blocking thymocyte adhesion to E-5 cells, allowed us to further demonstrate the specificity of thymocyte adhesion to PVC-immobilized E-5gp. When the adhesion assay was performed in presence of 100 μg/ml C3C12, thymocyte binding to immobilized E-5gp was completely abolished (figure 4). Furthermore, the same concentration of C3C12 $F(ab)'_2$ fragments was equally efficient in thymocyte binding inhibition, whereas a isotype-matched control mAb (MT4) had little inhibitory effect. These observations confirm that the reported thymocyte adhesion to immobilized E-5gp is mediated by the E-5 adhesion molecule which will be described in chapters 3 and 4.

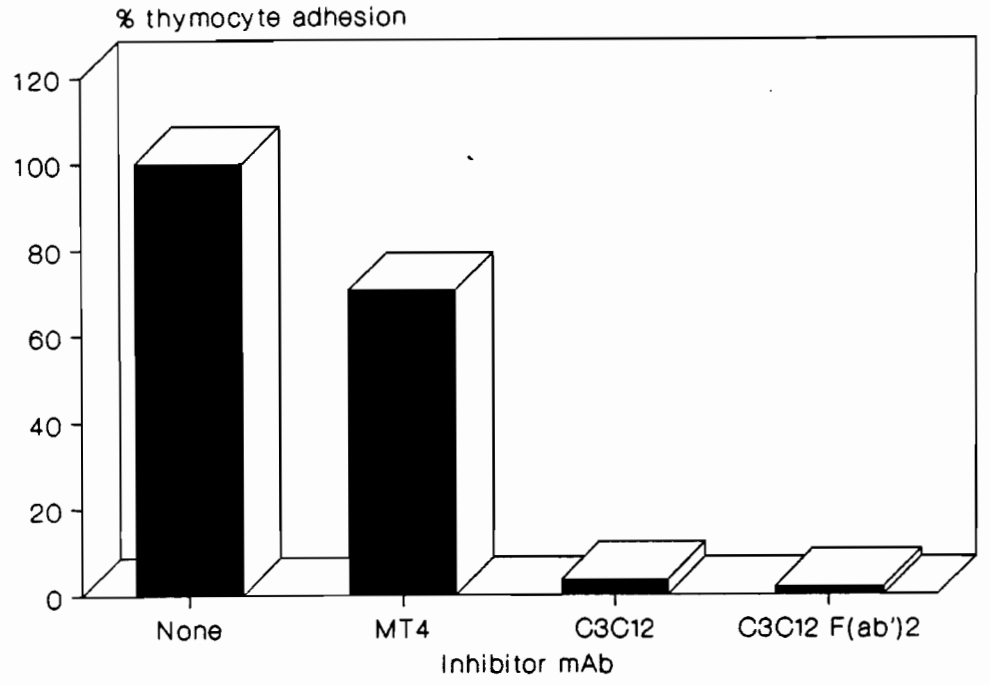


**Figure 4:** <u>Blocking of thymocyte adhesion to PVC-immobilized E-5gp by the C3C12 monoclonal antibody.</u> Chromium-labeled thymocytes were incubated with immobilized E-5gp in presence of 100 μg/ml of either the adhesion-blocking mAb C3C12 (chapter 4), its $F(ab')_2$ fragment, or the isotype-matched control mAb MT4. The adhesion observed in each case is compared with the adhesion measured in absence of inhibitor, which was given a arbitrary value of 100%.

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# CHAPTER 3

## A NOVEL THYMIC EPITHELIAL ADHESION MOLECULE.

This chapter describes the identification of the E-5 adhesion molecule, made possible by the development of the adhesion-blocking L-43 mAb and of the solid-phase thymocyte adhesion assay. The E-5 adhesion molecule is composed of three subunits (gp23/45/90) and has the same adhesion specificity as the E-5 cells from which it originates.*

* This work was published in Eur. J. Immunol. 20:2769 (1990).

## 3.1 Abstract

Interactions between thymocytes and thymic stromal cells are responsible for positive and negative selection of T cells, their differentiation, maturation and proliferation. The signals required for these events to occur often necessitate close contact, and indeed adhesion, between the cell types involved. The identification of specific adhesion molecules in this context is therefore a vital first step to determine the nature of the signal they mediate or facilitate at a given stage of differentiation. In the present work we identify, isolate and partially characterize a ligand present on thymic medullary epithelial cells which selectively binds CD4+CD8+ thymocytes found primarily in the thymic cortex. This adhesion molecule is composed of two non-covalently associated glycoproteins of 23 kD and 45 kD respectively, both of which are needed to bind to thymocytes. The importance of the finding is that the ligand, in isolated immobilized form, binds the same thymocyte subset as the original epithelial cell line from which it was isolated. The CD4+CD8+ thymocyte subset is the precursor of single positive mature T cells; hence the putative biological activity of the ligand in question takes place at a pivotal stage of T cell differentiation.

## 3.2 Introduction

T cell differentiation, by and large, occurs in the thymus, where bone-marrow derived precursor cells interact with various stromal microenvironments (reviewed by Adkins et al., 1987 and by Fowlkes and Pardoll, 1989). Such interactions between lymphoid cells on the one hand and a number of stromal cells on the other (Reviewed by von Boehmer, 1988; Kappler et al., 1987 and Lorenz and Allen, 1989) hand are responsible for positive and negative selection of T cells , the acquisition and loss of specific surface markers (Savion et al., 1987 and Brightman et al., 1989), with the concommitant development of functional T cell subsets (Tatsumi et al., 1990). During differentiation, thymocytes interact sequentially with different stromal cells as has been shown from the analysis of lympho-stromal complexes isolated from the thymus (Kyewski, 1987). The transition between immature CD4+CD8+ (double positive) thymocytes in the cortex to CD4+CD8- and CD4-CD8+ cells in the medulla is clearly a key event, so that the observation that thymocytes forming complexes with a thymic medullary epithelial cell line, E-5 (Potworowski et al., 1986), are mainly double positive (with a few CD4+CD8- cells) was of particular significance (Hugo and Potworowski, 1989a). In light of the above considerations, identification of the molecules involved in the formation of this complex will provide a strong handle for the characterization of the

contact-dependent events occuring at that pivotal stage of differentiation. The medullary epithelial molecule responsible for adhesion to double positive thymocytes does not seem, a priori, to correspond to any previously described adhesion molecule (Hynes, 1987, Arnaout, 1990 and Siegelman, 1989): indeed, complex formation is more efficient at 4°C than at 37°C, and is Ca++/Mg++ independent. Furthermore, the putative adhesion molecule on the E-5 plasma membrane is a trypsin-sensitive glycoprotein, the glycosylated moiety of which is not involved in adhesion to thymocytes (Hugo and Potworowski, 1989b). In the present work, we report the identification, isolation, partial characterization and binding specificity of this novel adhesion molecule found both on E-5 cells and on thymic medullary epithelial cells in situ. It is recognized by monoclonal antibody L-43, is composed of two subunits of 23kD and 45kD respectively and binds specifically the same thymocytes as do the E-5 cells.

## 3.3 Materials and methods

### 3.31 Cell lines

The E-5 cell line was derived from a primary culture of a C57Bl/6 thymus treated with a chemical carcinogen (Potworowski et al., 1986). The phenotype of the E-5 cell is characteristic of thymic medullary epithelial cells. The E-7 mutant cell line

was obtained by limit dilution cloning of E-5 cells, and thymocytes do not adhere to it.

### 3.32 E-5 adhesion and adhesion-inhibition assay

E-5 cells were grown in four chamber Labtek plastic slides in a growth medium (one volume of medium 199 supplemented with Hank's salts and one volume of M.E.M. medium (Eagle) supplemented with Earle's salt), to which "Nu Serum" (Collaborative Research, Lexington, MA., USA) was added to a final concentration of 10%. These cultures were used in the E-5/thymocyte adhesion assay (Hugo and Potworowski, 1989a), which was performed at 4°C.

Monoclonal antibodies were affinity purified using protein G Sepharose chromatography (Akerstrom et al., 1985). The adhesion-inhibition assay was performed at 4°C as follows: E-5 cells were pre-incubated for 30 minutes with the indicated concentrations of each mAb prior to the adhesion assay. The results are expressed as the mean number of thymocytes adhered per 100 E-5 cells (% adhesion).

### 3.33 Flow cytometric analysis of L-43 mAb reactivity

E-5 and E-7 cells were grown to confluency in the medium described above and harvested in PBS containing 1% fetal

bovine serum and 10mM EDTA. These cells were analysed for reactivity with L-43 (140$\mu$g/$10^6$ cells) using a FITC-conjugated goat anti-rat antibody (Zymed) on an EPICS-C flow cytometer (Coulter electronics, Hialeah, FL, USA) equipped with a single argon laser. Controls included the reaction with MT4 (anti-CD4) mAb followed by the conjugate, and cells reacted only with conjugate. Dead cells were excluded from the analysis on the basis of forward light scatter.

## 3.34 Preparation of E-5 cell membrane extract and solid phase immunoprecipitation

The E-5 cell surface glycoprotein extract (E-5gp) was prepared by solubilization of E-5 membranes (obtained by nitrogen decompression of E-5 cells followed by differential centrifugation [Hunter and Commerford, 1960 and Islam et al., 1983]) in 50mM Tris, 150mM NaCl pH 8.0 containing 0.25% lauryl maltoside. This membrane extract was then passed through a lentil lectin sepharose column, and retained glycoproteins were eluted with 0.5M glucose. This E-5gp extract was dialysed against 0.1M $PO_4$, pH7.5, and 50$\mu$g were radio-labelled using the Iodo-gen (Salacinski et al., 1981) iodination reagent (Pierce). For the immunoprecipitation procedure (Tamura et al., 1984), 10$\mu$g of L-43 and MT4 (anti-CD4) monoclonal antibodies in 50$\mu$l PBS were plated in PVC plates (96 wells, Micro Test III, Falcon). After overnight incubation at 4°C,

the wells were washed twice with 300μl of PBS containing 0.5% NP-40 or PBS. After blocking the wells with 5% BSA in either PBS/0.5% NP-40 or PBS for 45 minutes at 25°C, an amount of 1μg radiolabeled E-5gp extract ($12x10^6$ cpm) in a volume of 50μl 1% BSA in PBS/0.5% NP-40 or PBS, was pre-adsorbed by incubation with anti-CD4 mAb for 90 minutes at 25°C, and directly transfered into wells containing immobilized L-43. For the competition study, radiolabelled E-5gp was incubated for 45 minutes in presence of 100μg L-43 in a volume of 50 μl PBS, prior to incubation with immobilized MT4 and L-43. After a 90 minute incubation at 25°C, unbound proteins were removed by extensive washing with PBS/0.5% NP-40 or PBS, and retained material was eluted using 50μl of either reducing (R) or non-reducing (NR) SDS sample buffer. Electrophoresis of precipitated proteins was performed according to standard procedures (Laemmli, 1970), using 10% acrylamide gels. After staining with Coomasie Brilliant Blue R250, the gels were dried and exposed for 15 days with a Kodak X-Omat AR film.

## 3.35 Purification of individual glycoproteins

The E-5 glycoproteins (obtained as described in section 2.4) were resolved by electrophoresis under non-reducing conditions on 12% polyacrylamide gels. The gels were then briefly stained with 0.3M $CuCl_2$ (Lee et al., 1987), and the bands corresponding to gp23, gp45 and gp90 were excised. When

rerun on a 10% SDS-gel, all three glycoproteins resolved as single, well-defined bands (not shown). After chelating the $CuCl_2$ for 15 minutes with 250mM Tris-HCl pH 9.0 containing 250mM EDTA, each glycoprotein was electroeluted at 150v for 16 hours in 25mM Tris, 192mM glycine pH 8.3, containing 0.1% SDS (Donofrio et al., 1986). SDS was removed by passing eluted proteins through a 1 mL extracti-gel D column (Pierce) equilibrated with PBS, and proteins were concentrated by ultrafiltration using a CX-10 filter (Millipore).

## 3.36 Adhesion of $^{51}Cr$-labelled thymocytes to purified E-5 glycoproteins

Similar amounts of purified gp23, gp45 and gp90 were plated in PVC plates. In these experiments, a low molecular weight glycoprotein (gp18), also present in the E-5gp extract, was purified using the same methods and used as a negative control. In addition, gp23 and gp45 were plated together in the same well, each glycoprotein being present at half the concentration used for each individually plated protein. Proteins were incubated for 90 minutes at 25°C and then at 4°C overnight (Kane et al., 1989). Each well was washed three times with 250$\mu$l PBS and blocked 90 minutes with PBS containing 5% BSA. After blocking, $5x10^5$ $^{51}Cr$-labeled thymocytes were added to each well in a volume of 55$\mu$l calcium and magnesium-free PBS containing 2% BSA and the plates were

incubated 90 minutes at 4°C, which correspond to the conditions used to detect thymocyte adhesion to E-5 cells. Non-adhered thymocytes were removed by five consecutive washes with 250µl PBS. Adhering thymocytes were lysed with 2% SDS and the supernatant counted by a Gamma 7000 counter (Beckman).

## 3.37 Cell sorting

$50x10^6$ thymocytes were labelled using 500µCi $Na_2{}^{51}CrO_4$ (ICN Biomedicals) and washed four times with 15ml PBS. Labelled thymocytes were then reacted with phycoerythrin-conjugated anti-mouse CD4 mAb (Becton Dickinson, final dilution 1/2) and with a FITC-conjugated rat anti-mouse CD8 mAb (final dilution 1/10). Double-labelled thymocytes were then sorted using the same aparatus described in section 2.3. Preliminary experiments indicated that incorporated $^{51}Cr$ does not interfere with the sorting (not shown). All four populations could be isolated with the following degrees of purity: CD4+8+: 96%; CD4+8-: 90%; CD4-8+: 74% (contaminating cells were double negatives); CD4-8-: 90%.

## 3.4 Results

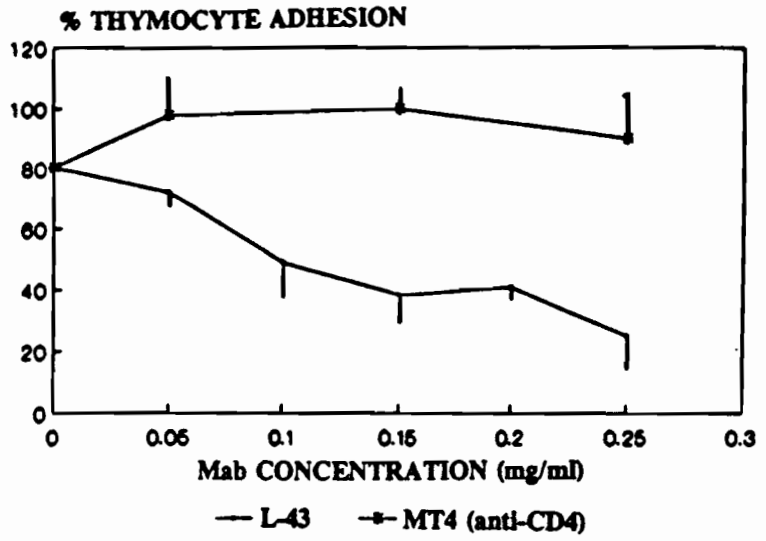
### 3.41 L-43 mAb blocks thymocyte adhesion to E-5 cells

The approach chosen to identify the E-5 adhesion molecule was to raise rat monoclonal antibodies against E-5gp, a surface glycoprotein extract of E-5 cells. E-5gp was selected as an immunogen on the basis of its ability to block adhesion between E-5 cells and thymocytes, when added to the reaction mixture (Hugo et al., 1989). Monoclonal antibody L-43 was selected both for its immunofluorescence reactivity with medullary epithelial cells in frozen sections of mouse thymus, and for its ability to block E-5/thymocyte adhesion. Purified L-43 antibody blocked the adhesion reaction in a dose-dependent manner in the concentration range of 0.1 to 0.25 mg/ml (figure 1a). To rule out any possible Fc receptor-mediated inhibition on E-5/thymocyte adhesion, an isotype-matched, affinity-purified irrelevant mAb was used as a control. Steric inhibition was also ruled out by using other E-5 reactive mAbs (not shown).

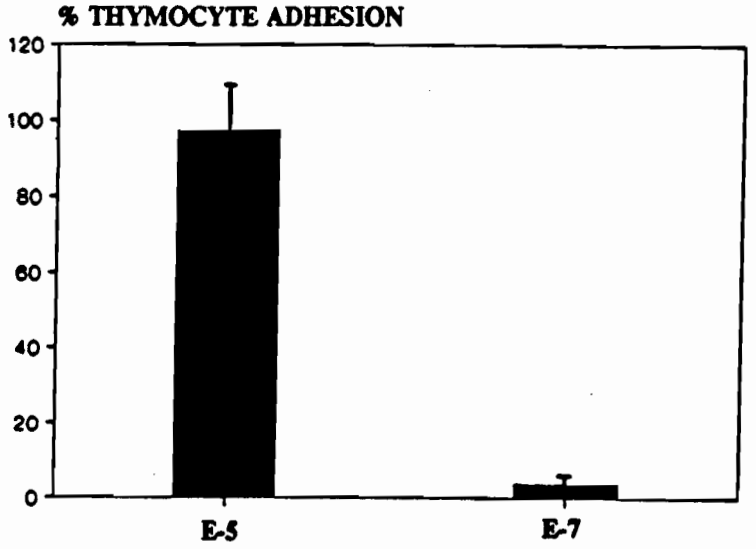
As a further indication that L-43 was indeed directed to the E-5 adhesion molecule, the antibody was reacted both with E-5 and with E-7, a non-adhering E-5 mutant. By flow cytometry, the antigen recognized by L-43 was found to be highly expressed by E-5 cells and only marginally by E-7. In

**Figure 1:** Properties of L-43 rat monoclonal antibody recognizing the putative E-5 adhesion molecule. a) Adhesion inhibition assay using purified L-43 and an isotype-matched control antibody, MT4 (anti-CD4), showing a dose-dependent inhibition of thymocyte/E-5 adhesion by L-43, but not by the control mAb. b) Adhesion of thymocytes to E-5 and to E-7 cells, showing the total inability of the E-7 clone to form complexes with thymocytes. c) Flow cytometric analysis of L-43 antigen expression by both E-5 and E-7 cell lines, demonstrating that the reactivity of L-43 mAb is closely associated with the adhesion activity of both cell lines (shown in b).

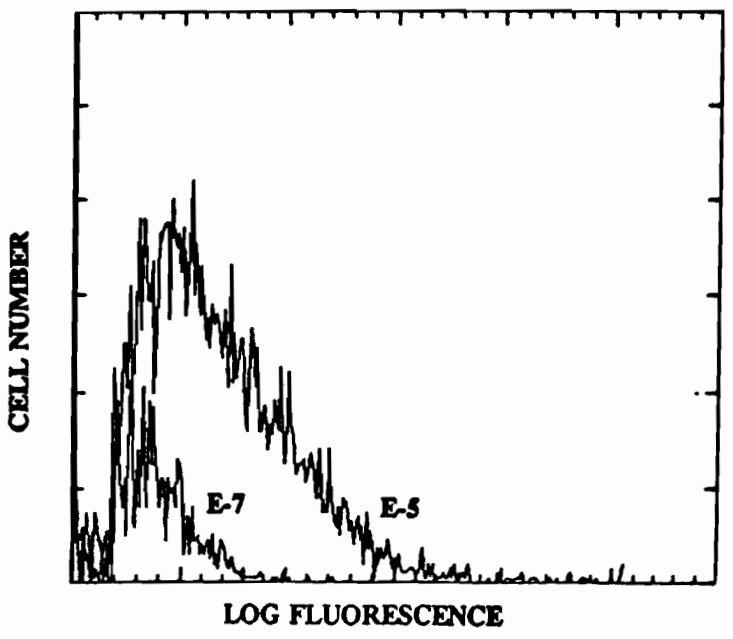
a)


% THYMOCYTE ADHESION
120
100
80
60
40
20
0
0
0.05
0.1
0.15
0.2
0.25
0.3
Mab CONCENTRATION (mg/ml)
L-43
MT4 (anti-CD4)

b)


% THYMOCYTE ADHESION
120
100
80
60
40
20
0
E-5
E-7

c)


CELL NUMBER
E-7
E-5
LOG FLUORESCENCE

fact, 5.3 times more E-5 cells were found to express the L-43 antigen. Furthermore, positive E-5 cells had a surface density of L-43 antigen 5.4 times higher than positive E-7 cells. This correlates with the adhesion potential of both cell lines (fig 1, b and c).

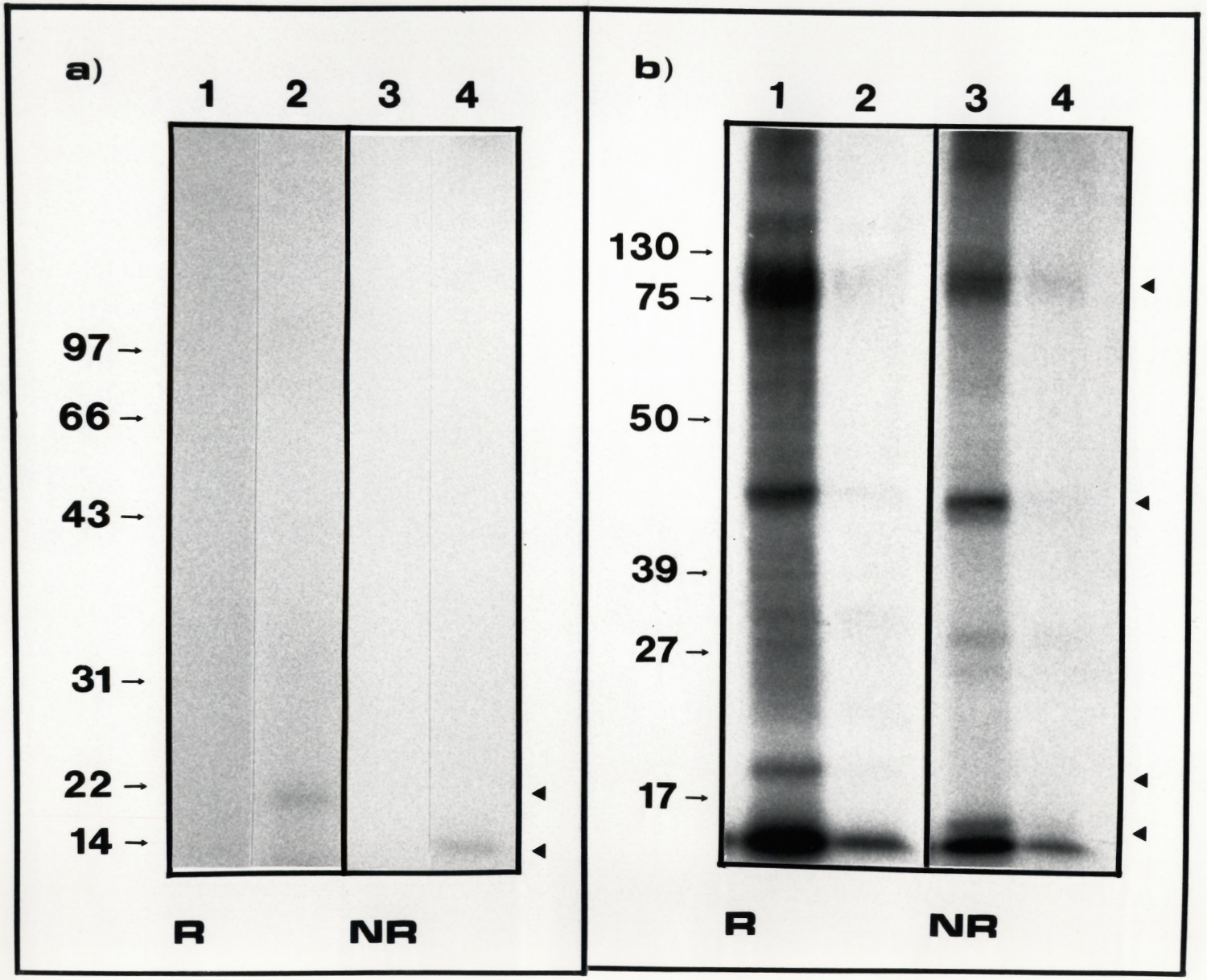
### 3.42 L-43 mAb recognizes a multimeric complex

Using solid-phase immunoprecipitation of radio-iodinated E-5 gp with mAb L-43, a 23kD glycoprotein (gp23) was precipitated (figure 2a, lane 2). Under non-reducing conditions, gp23 was found to have a higher mobility (figure 2a, lane 4), indicating the presence of intrachain disulfide bonds (McIntyre et al., 1989).

To determine whether gp23 was responsible for thymocyte adhesion to E-5 cells, the following approach was used: gp23 was purified by SDS-PAGE and electroelution and renatured by removal of SDS; the purified glycoprotein was then immobilized into PVC plates and assayed for its capacity to mediate adhesion of chromium-labelled thymocytes. In these experiments, gp23 was found to be inactive, wheras crude E-5 glycoprotein extract was highly adhesive (data not shown).

We therefore investigated the possibility that gp23 could be part of a multimeric receptor responsible for

**Figure 2:** Solid-phase immunoprecipitation of radiolabelled E-5gp extract using adhesion-blocking antibody L-43. Immunoprecipitation was performed in PBS containing 0.5% NP-40 (a) or in PBS alone (b), using a method described elsewhere (Tamura et al., 1984). Panel a) shows the proteins precipitated by control mAb MT4 (lanes 1 and 3) and by mAb L-43 (lanes 2 and 4), under both reducing (R) and non-reducing (NR) conditions. Panel b) shows the proteins retained by L-43 mAb (lanes 1 and 3) and the disapearence of all three gp23, gp45 and gp90 resulting of a pre-incubation of iodinated E-5gp extract with 100$\mu$g of L-43 prior to incubation with immobilized L-43 mAb (lanes 2 and 4). The respective positions of gp23, gp45 and gp90 are indicated by arrows and molecular weights ($x10^{-3}$) are indicated on the left of each panel.


a)
1
2
3
4
97
66
43
31
22
14
R
NR
b)
1
2
3
4
130
75
50
39
27
17
R
NR

E-5/thymocyte adhesion. To identify putative gp23-associated glycoproteins, further immunoprecipitations were performed, this time in the absence of NP-40. Under these conditions, L-43 precipitated gp23, as well as two associated glycoproteins, gp45 and gp90 (figure 2b). The incubation of radio-labeled E-5gp extract with excess L-43 prior to precipitation resulted in the total disappearance of all three bands, indicating that all three glycoproteins are part of a same complex.

### 3.43 The association of gp23 and gp45 subunits is necessary for thymocyte adhesion

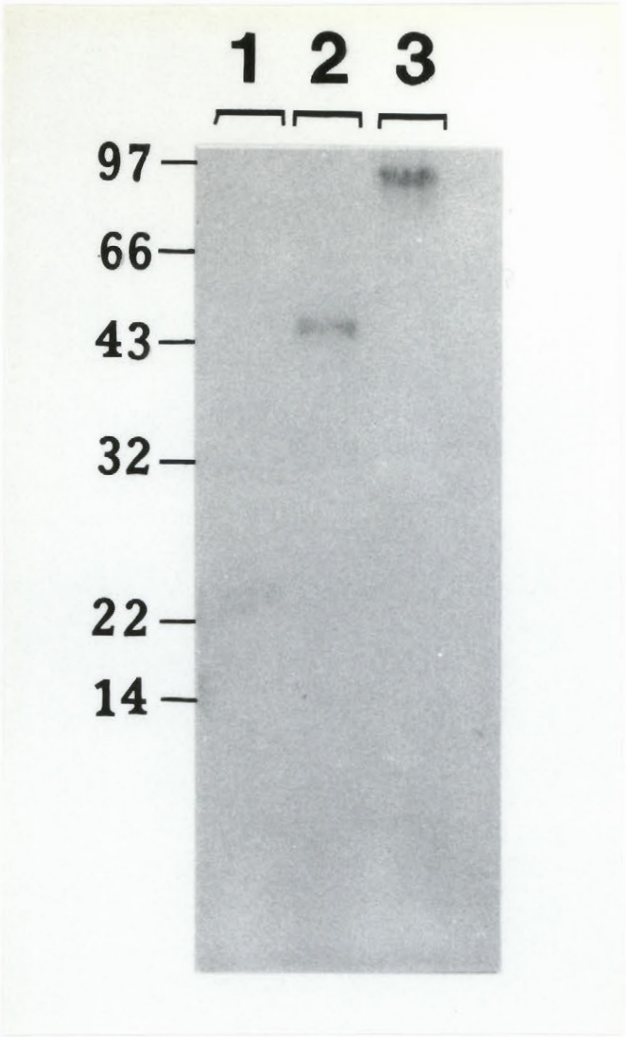
The three glycoproteins (gp23, gp45 and gp90) precipitated by mAb L-43 were purified (figure 3a), immobilized and tested for their ability to mediate thymocyte adhesion. Individually, all three glycoproteins were found to be negative. However, when gp23 and gp45 were plated together, a marked adhesion of thymocytes was observed (figure 3b).

### 3.44 Double positive thymocytes adhere to the purified E-5 adhesion complex

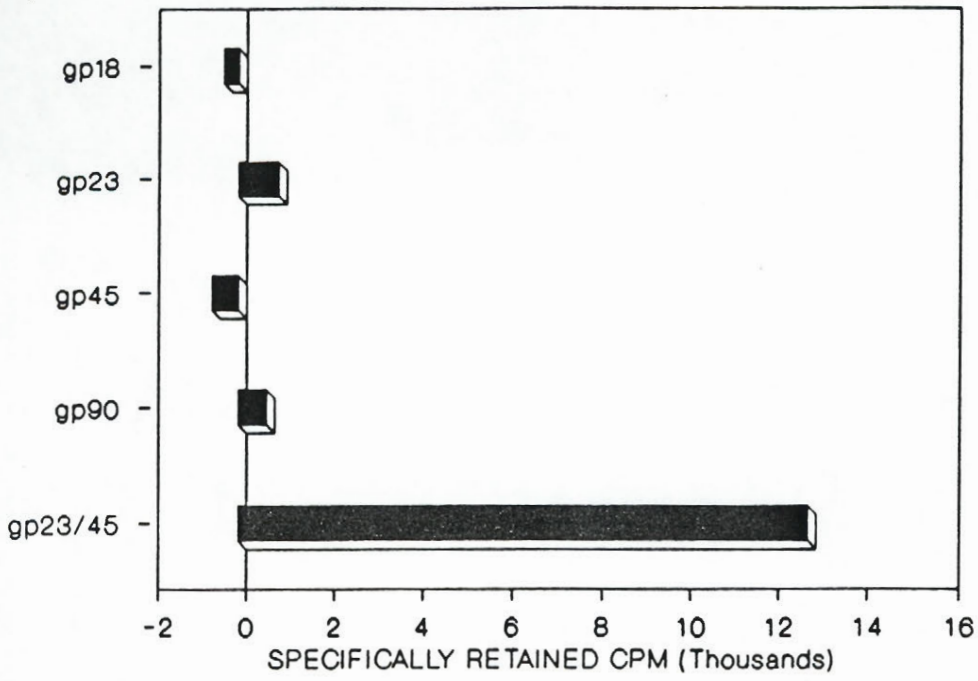
$^{51}Cr$-labelled thymocytes were sorted into double positive, double negative and single positive cell populations, with respect to CD4 and CD8 expression, and each population ($7x10^4$ cells per assay) was tested for adhesion to

**Figure 3:** Adhesion of $^{51}Cr$-labelled thymocytes to purified and immobilized E-5 glycoproteins. a) gp23, gp45 and gp90 were purified by immunoprecipitation, seperately electroeluted and subjected to 10% SDS-PAGE. b) gp23, gp45 and gp90 were immobilized into PVC wells, and tested individually for their ability to mediate thymocyte adhesion. As a control, a low molecular weight glycoprotein, gp18, also present in the E-5gp extract, was used. Finally, gp23 and gp45 were plated together in a 1:1 (v/v) ratio, in the same final volume used for the other samples.

a)


1 2 3
97
66
43
32
22
14

b)


gp18
gp23
gp45
gp90
gp23/45
-2
0
2
4
6
8
10
12
14
16
SPECIFICALLY RETAINED CPM (Thousands)

purified gp23 and gp45 ,with or without gp90, immobilized into PVC wells. In these experiments, 0.1μg of each purified glycoprotein was used. Only double positive thymocytes and a few CD4+CD8- thymocytes were found to adhere to the gp23/gp45 complex (table 1). This binding was efficiently blocked by pre-incubating the immobilized glycoproteins with 10μg L-43 mAb prior to plating the sorted cells. In the same conditions, the MT4 control mAb had no inhibitory effect. The addition of gp90 to the gp23/45 complex neither increased nor decreased adhesion, indicating that this molecule is not directly involved in the adhesion process.

**Table 1.** Adhesion of isolated thymocyte populations to purified and immobilized E-5 glycoproteins*.

| Thymocytes | inhibitory mAb | gp23/gp45 | gp23/gp45/gp90 |
|---|---|---|---|
| CD4+8+ | ---- | 52.5 ± 0.8 | 51.7 ± 1.0 |
| | L-43 | 10.4 ± 4.0 | 12.7 ± 2.2 |
| | MT4 | 49.2 ± 7.2 | ND |
| CD4+8- | ---- | 4.2 | ND |
| CD4-8+ | ---- | 0.0 | ND |
| CD4-8- | ---- | 0.0 | ND |

*expressed as $\frac{\text{retained cpm}}{\text{plated cpm}} \times 100$

## 3.5 Discussion

The properties of the interaction of thymocytes with E-5 cells was striking in that they ruled out the involvment of previously described adhesion molecules. Moreover, the specificity of E-5 cells for double positive thymocytes has no equivalent in any other system (Hynes, 1987, Arnaout, 1989, Siegelman et al., 1989, Yednock and Rosen, 1989 and Patarroyo and Makgoba, 1989). It was therefore important to identify and purify this E-5 adhesion molecule, which would have adhesion properties and specificities similar to those of E-5 cells.

Using an adhesion-blocking rat mAb, L-43, it was possible to identify this E-5 adhesion molecule, whose expression is closely related to the thymocyte adhesion potential of both E-5 and E-7 (thymocyte non-adhering) cells. L-43 recognizes a gp23, which was unable to mediate thymocyte adhesion by itself. Under milder conditions of immunoprecipitation, gp23 was found to be associated with two other glycoproteins, namely gp45 and gp90. Additional experiments revealed that the gp23 subunit needs to be non-covalently associated to the gp45 subunit in order to be adhesive to thymocytes. The role of gp90 is at present unknown. It could be a precursor of gp45, in which case peptide mapping should provide useful information. It could also be an accessory molecule which

takes part, for instance, in message transmission, once the adhesion has taken place.

The thymocytes adhering to the gp23/45 complex (half of the double positive population plus a small minority of CD4+ single positives) correspond to the pattern of thymocytes adhering to E-5 cells (Hugo and Potworowski, 1989a). Furthermore, both this molecular adhesion to double positive thymocytes as well as the E-5 cellular adhesion to the same cells were clearly inhibited by L-43 mAb. Taken together, these results show that the molecules described in this paper constitute the ligand present on E-5 cells, and indeed on medullary epithelial cells in situ, if one considers the immunofluorescence reactivity of L-43 on thymus frozen sections.

The physical structure of this adhesion molecule is, to the best of our knowledge, different from any known epithelial adhesion molecule involved in lymphocyte/epithelial (Hynes, 1987, Arnaout, 1989, Siegelman et al., 1989, Yednock and Rosen, 1989 and Patarroyo and Makgoba, 1989), or even epithelial/epithelial cell interactions (Obrink, 1986), most of which are calcium dependent. Its low molecular weight and functional independence from divalent cations, both on intact E-5 cells[15] and in purified form (section 2.6), clearly indicates the uniqueness of this adhesion molecule.

Furthermore, its specificity for double positive thymocytes and its restricted expression by thymic medullary epithelial cells, argue for a role of this adhesion molecule in the stepwise process of intrathymic T lymphocyte differentiation. Work is now in progress to determine which signal, if any, is mediated or facilitated by this adhesion molecule to double positive thymocytes.

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# CHAPTER 4

## TYROSINE KINASE ACTIVATION IN THYMIC EPITHELIAL CELLS: necessity of thymocyte contact through the gp23/45/90 adhesion complex.

In chapter 3, we have identified the E-5 adhesion molecule as a multimeric complex of three glycoproteins. Both gp23 and gp45 were shown to be involved in the adhesion of $CD4^+CD8^+$ thymocytes. The third subunit, however, is not involved in thymocyte adhesion, and its role is unknown. In this chapter, we report that thymocyte adhesion through gp23/45 leads the stimulation of E-5 cells and to the phosphorylation of gp90 on tyrosine residues by a tyrosine kinase physically associated to the adhesion complex. A putative role of gp90 as a protein tyrosine kinase is discussed.*

* This work was accepted for publication in Eur. J. Immunol. (july 1992).

## 4.1 Abstract

Interactions between thymocytes and thymic stromal cells are necessary for T cell differentiation, maturation and proliferation. The signals required for these events to occur often necessitate close contact, and indeed adhesion, between the cell types involved. While the transmission of signals from stromal cells to thymocytes has been well documented, there is little evidence that binding of thymocytes to stromal cells can result in stromal cell activation. We have recently identified a novel thymic epithelial adhesion complex composed of three non-covalently associated glycoproteins (gp23, gp45 and gp90). While gp23 and gp45 are jointly required for adhesion to thymocytes, the function of gp90 is unknown. In the present work, we show that gp23/45-mediated contact with thymocytes induces *de novo* tyrosine phosphorylation of gp90. Furthermore, the protein tyrosine kinase responsible for gp90 neophosphorylation is itself an integral part of the adhesion complex.

## 4.2 Introduction

Thymic stromal cells, comprising various epithelial cells, macrophages and dendritic cells, are all known to form complexes with developing thymocytes (Kyewski, 1987). A number of surface molecules involved in the formation of such complexes are directly responsible for signal transduction to thymocytes. For exemple, stromal MHC interaction with thymocyte TCR results either in clonal deletion of auto-reactive thymocytes or in positive selection (Teh et al., 1988, Blacjman et al., 1990 and Lorenz and Allen, 1989). In addition, adhesion molecules such as CD2 and LFA-1 are expressed by developing thymocytes and are known to generate intracellular signals upon interaction with their stromal ligands, namely LFA-3 and ICAM-1 (Bierer et al., 1988, He et al., 1988, van Noesel et al., 1988 and Kuijpers et al., 1990).

While the transmission of signals from stromal cells to thymocytes is well documented (Denning et al., 1988 and Savion et al., 1989), there is only scanty and indirect evidence that the binding of thymocytes to stromal cells can result in stromal cell activation. Le et al. (1990) have shown that thymocyte binding to thymic epithelial cells results in a LFA-3-dependent increase in IL-1 production. However, the mechanism of signal transduction is unknown. Shores et al. (1991), using SCID mice, have shown that only $TCR^+$ thymocytes

could induce the normal organization and maturation of thymic medullary epithelial cells. At this time, the dependence of this phenomenon on lympho-epithelial contact and signal transduction can only be assumed.

These instances raise the intriguing possibility that thymic epithelial cells not only are regulators of T cell differentiation but that they can themselves be "regulated" by thymocytes.

We have recently identified, isolated and partly characterized a thymic epithelial adhesion molecule from the E-5 thymic epithelial cell line which is unique in that it selectively binds $CD4^{+}CD8^{+}$ thymocytes, at both 37°C and 4°C, independently of $Ca^{++}$ and $Mg^{++}$ (Couture et al., 1990). This adhesion molecule is composed of two non-covalently associated glycoproteins of 23kD and 45kD (gp23 and gp45) respectively, both of which are needed to bind thymocytes. Monoclonal antibodies L-43 and C3C12, recognizing the gp23 subunit, can co-precipitate gp23, gp45 as well as an additional glycoprotein, gp90. This trimeric complex will be referred to as the E-5 "adhesion complex". The gp90 subunit is not necessary for thymocyte adhesion and its role is unknown.

In the present work, we show that contact of E-5 epithelial cells with thymocytes induces phosphorylation of at

least three epithelial substrates, including gp90. The phosphorylation of gp90 occurs exclusively on tyrosine residues, possibly via autophosphorylation. This report thus presents the first direct evidence of signal transduction in thymic epithelial cells triggered by contact with thymocytes.

## 4.3 Materials and methods

### 4.31 Cells and Antibodies.

The E-5 cell line was derived from a primary culture of C57BL/6 mouse thymuses immortalized with a chemical carcinogen, n-methyl-n-nitro-n-nitrosoguanidine (Potworowski et al., 1986). E-5 cells were grown in a 1:1 mixture of medium 199 supplemented with Hank's salt and of MEM (Eagle) supplemented with Earle's salt, containing 10% "Nu Serum" (Collaborative Research, Lexington, MA). They have been characterized as medullary epithelial cells, on the basis of reactivity with a battery of mAbs (Potworowski et al., 1986). The NG-3 cell line was derived from C57BL/6 mouse thymuses by treatment with collagenase/dispase followed by sedimentation on an FCS gradient (Kyewski et al., 1982). These cells have been co-transfected with SV40 *ori*$^-$ mutant 6-1 and the neomycin resistant gene pSV2Neo, using calcium phosphate. While not fully characterized, NG-3 cells do not express gp23, as determined by reactivity with the C3C12 mAb

(unpublished); they will be used as a negative control, instead of the E-7 clone used in previous work (Couture et al., 1990) which was recently found to be unstable with respect to gp23 expression. The NG-3 cells were grown in the same medium as E-5 cells. The Ti-6 cell line was derived from a radiation-induced thymic lymphoma in C57BL/6 mice (Sankar-Mistry and Jolicoeur, 1980). Ti-6 cells are $CD4^{-}CD8^{lo}$ ,but they have been shown to form complexes with E-5 via the same epithelial adhesion complex as CD4+CD8+ thymocytes (Hugo et al., 1989), hence their interest in the present study. Ti-6 cells were grown in RPMI-1640 supplemented with 10% FCS. Syngeneic thymocytes were prepared fresh for each experiment.

The C3C12 mAb was produced by a subclone of the L-43 hybridoma, already described (Couture et al., 1990). Like L-43, C3C12 blocks the adhesion of thymocytes and Ti-6 to E-5 cells, recognizes the gp23 subunit of the E-5 adhesion complex and, in absence of detergent, can co-precipitate gp45 and gp90 from a lentil lectin-binding E-5 glycoprotein extract. C3C12 was purified from ascitic fluid raised in Lou X AO F1 rats by consecutive precipitations with caprylic acid (Russo et al., 1983) and ammonium sulfate. The MT4 (anti-CD4), a rat IgG2a, was purified from culture supernatant by protein G chromatography, and served as an isotype-matched control for C3C12.

**4.32 Phosphorylation assay.**

The method developed by Shin et al. (1990) has been used. Briefly, E-5 cells were grown to confluency in 60 mm Petri dishes (Corning), washed and incubated at 37°C for 60 min in a phosphate-starving medium (150 mM NaCl, 5 mM MgCl2, 5 mM KCl, 2 mM glutamine, 1.8 mM glucose, 10 mM Tris-acetate, pH 7.4 containing 2% FCS dialysed against deionised water). After addition of 50 μCi [$^{32}$P]-Orthophosphate (ICN), 2.5 ml of a phosphate-starved thymocyte suspension were added to the above E-5 culture at a final thymocyte concentration of 4 x $10^6$/ml and the cultures further incubated at 37°C for 30 min (unless otherwise specified). For phosphorylation inhibition, 10 μg/ml genistein (Akiyama et al., 1987) were added to the starving medium and kept during both starving and co-culture with thymocytes. We have verified that addition of genistein in concentrations of up to 50 μg/ml had no effect on the viability of E-5 cells in our experimental conditions. [$^{32}$P]phosphate incorporation was stopped by adding cold PBS containing 1 mM sodium orthovanadate. Adhered thymocytes were forcibly removed by extensive washing of E-5 cells with PBS-vanadate, following which the absence of thymocytes was verified by phase contrast microscopy. Thymocyte-free E-5 cells were then lysed at 4°C by treatment with 1 ml 1% NP-40 in 20 mM Tris-HCl pH 8.0 containing 137 mM NaCl, 10% glycerol, 1 mM $Na_3VO_4$, 2 mM EDTA, 1 mM PMSF, 20 μM leupeptin and 0.15

U/ml aprotinin (Gold et al., 1990) and insoluble material removed by centrifugation at 15000g. Aliquots of these lysates were either subjected to 8.75% SDS-PAGE in non-reducing conditions and auto-radiography or used for immunopurification. Each phosphorylation experiment was repeated at least 3 times. The problem of repeating phosphorylation experiments using rapidly decaying $^{32}P$, (thus obtaining progressively smaller CPM counts with each repetition) has been dealt with by presenting the data as the means and standard deviation of the ratios of experimental CPM over control CPM (E-5 cells alone). As a consequence, the value assigned to the phosphorylation ratio for unstimulated E-5 cells has necessarily a value of 1.

## 4.33 Immunopurification of the gp23/45/90 adhesion complex.

Identical amounts (in $\mu$g of protein) of phosphorylated E-5 cell lysates from various experimental preparations were incubated 2 hrs at 4°C with 100 $\mu$l of lentil lectin Sepharose 4B (Pharmacia). After three washes with detergent-free lysis buffer, retained material was eluted with 5% glucose in washing buffer. This step is necessary to remove the detergent from lentil lectin-bound proteins in order to allow the co-precipitation of all three subunits (gp23,45 and 90) of the E-5 adhesion complex using the C3C12 mAb. The solid-phase immunoprecipitation technique used to co-precipitate gp23,

gp45 and gp90 has been described in details elsewhere (Couture et al., 1990). Briefly, lentil lectin eluates were transfered to PVC plates coated with 10 μg of C3C12 mAb. After extensive washing with PBS-vanadate, antibody-bound proteins were eluted with SDS-containing sample buffer for SDS-PAGE analysis.

**4.34 Co-culture system.**

E-5 cells were grown to confluency in the lower compartment of 24.5 mm Transwell cell culture chambers (Costar, Cambridge, MA, model 3412) and starved as described. Phosphate-starved thymocytes or Ti-6 cells (4x $0^6$/ml in 2.5 ml starving buffer) were placed in the upper compartment, the bottom of which is a polycarbonate filter with 0.4 μm pores. When in position, the filter supporting the lymphoid cells is 1 mm above the floor of the lower compartment on which the epithelial cells were grown. After 30 min of equilibration, 250 μCi of [$^{32}$P]orthophosphate were added to the lower chamber. After 30 min of co-culture at 37°C, the E-5 cells were washed and lysed as described. The incorporation of [$^{32}$P]-phosphate into E-5 proteins was estimated by scintillation counting of 1 μg of each cell lysate. As a way to controle for a possible adsorption of secreted molecules onto the filter, "upside down" cultures were carried out, in which E-5 cells grown on a glass coverslip were suspended upside down, separated from lymphoid cells by 2 mm of medium.

This more cumbersome approach proved valuable to demonstrate the validity of the insert system and the data are therefore not reported.

## 4.35 Phosphoamino acid analysis.

Immunoprecipitates were eluted from C3C12-coated PVC plates with 0.1 M glycine, pH 2.3 and precipitated with 20% TCA for 60 min at 4°C. After centrifugation at 15 000g, the pellets were washed three times with ethanol and dissolved in 300 µl 6 N constant boiling HCl (Pierce). Acid hydrolysis was performed at 110°C for 60 min (Sefton and Hunter, 1981). Separation of hydrolysed phosphoamino acids was done by two dimensional thin layer electrophoresis on 20 x 20 cm cellulose plates (100 µm, Sigma), essentially as described (Sefton and Hunter, 1981 and Cooper et al., 1983), except that the pH 1.9 run was carried out at 750 volts for 3 hrs and the pH 3.5 run at 300 volts for 3 hrs. Individual phosphoamino acids were identified by staining internal standards (O-phospho-L-Serine, O-Phospho-L-Threonine and O-Phospho-L-Tyrosine, Sigma, St-Louis, Mo) with 2% ninhydrin in ethanol.

## 4.36 In vitro immune complex kinase assay.

For the detection of in vitro kinase activity, the E-5 cells were co-cultured with thymocytes after phosphate-

starvation of both cell types as described earlier, but in absence of [$^{32}$P]Phosphate. The gp23/45/90 adhesion complex was purified by precipitation of identical amounts of each cell lysate first with lentil lectin Sepharose. The material eluted with 5% glucose was transfered to C3C12 mAb-coated PVC plates, previously blocked with 5% BSA. After extensive washing with detergent-free lysis buffer, C3C12-bound gp23/45/90 was incubated with 50 $\mu$Ci [$\gamma$-$^{32}$P]ATP in 10 mM $MnCl_2$, Tris-HCl pH 7.4 for 30 min at 25°C, as recently described (Kaplan et al., 1991). This [$\gamma$-$^{32}$P]ATP incubation step was carried out either in absence or in presence of genistein (10 $\mu$g/ml), a tyrosine kinase inhibitor. Phosphorylated proteins were eluted with SDS containing sample buffer and analysed by 12% SDS-PAGE.

## 4.4 Results

### 4.41 gp23/45-triggered phosphorylation of E-5 protein substrates.

E-5 cell activation resulting from interaction with either thymocytes or Ti-6 cells was investigated by incorporation of [$^{32}$P]phosphate into E-5 cellular proteins (figure 1a). When stimulated by a 30 min thymocyte contact, the level of [$^{32}$P]phosphate incorporation per $\mu$g of protein from E-5 cells was 3.2 fold higher ($n=8$, $p<0.001$ using a paired Student t test) than that of E-5 cells cultured alone in the same conditions. Similarly, a 2.9 fold increase was observed when E-5 cells were stimulated by a 30 min Ti-6 contact ($n=4$, $p<0.01$). To ensure that it was through the gp23/45 adhesion molecule that the observed increased [$^{32}$P]phosphate incorporation was generated, anti-gp23 mAb C3C12 (100 $\mu$g/ml) was used to block gp23/45-mediated interactions. This blockage resulted in the abolition of both thymocyte and Ti-6 induced phosphorylation of E-5 cells, whereas a similar treatment with an isotype control mAb (MT4) had no inhibitory effect on E-5 stimulation (figure 1a). The gp23/45 dependence of epithelial cell activation was further demonstrated by the observation that increased phosphorylation was not induced by thymocyte co-culture with NG-3 thymic stromal cells, which are gp23$^-$ (not shown).

**Figure 1:** Induction of E-5 epithelial phosphorylation by gp23/45-mediated contact with thymocytes or Ti-6 cells. a) [$^{32}$P]phosphate incorporation per $\mu$g of E-5 cell lysates. The vertical axis represents the ratio between the cpm counts from each phosphorylation experiment to the cpm counts of E-5 cells alone (1 ± 0 by definition). E-5: E-5 cells cultured alone; E-5 + Thy: E-5 cells after 30 min. contact with thymocytes; E-5 + Ti-6: E-5 cells after 30 min. contact with Ti-6 cells. Note that blockage of E-5 phosphorylation is achieved by the addition of the adhesion-blocking anti-gp23 mAb C3C12, but not by the isotype-matched controle mAb MT4. The number of independent observations (n) is indicated for each experiment.

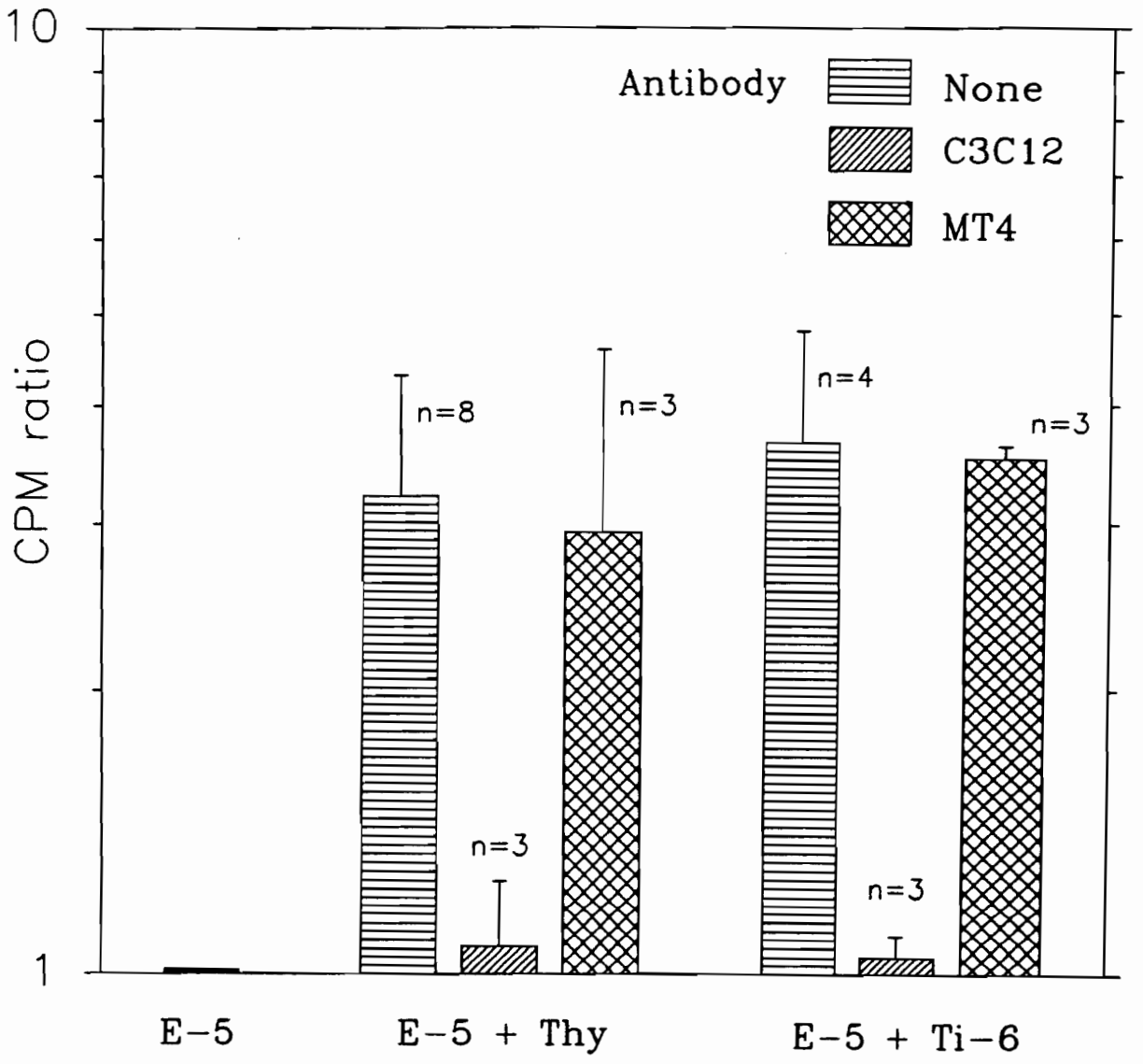
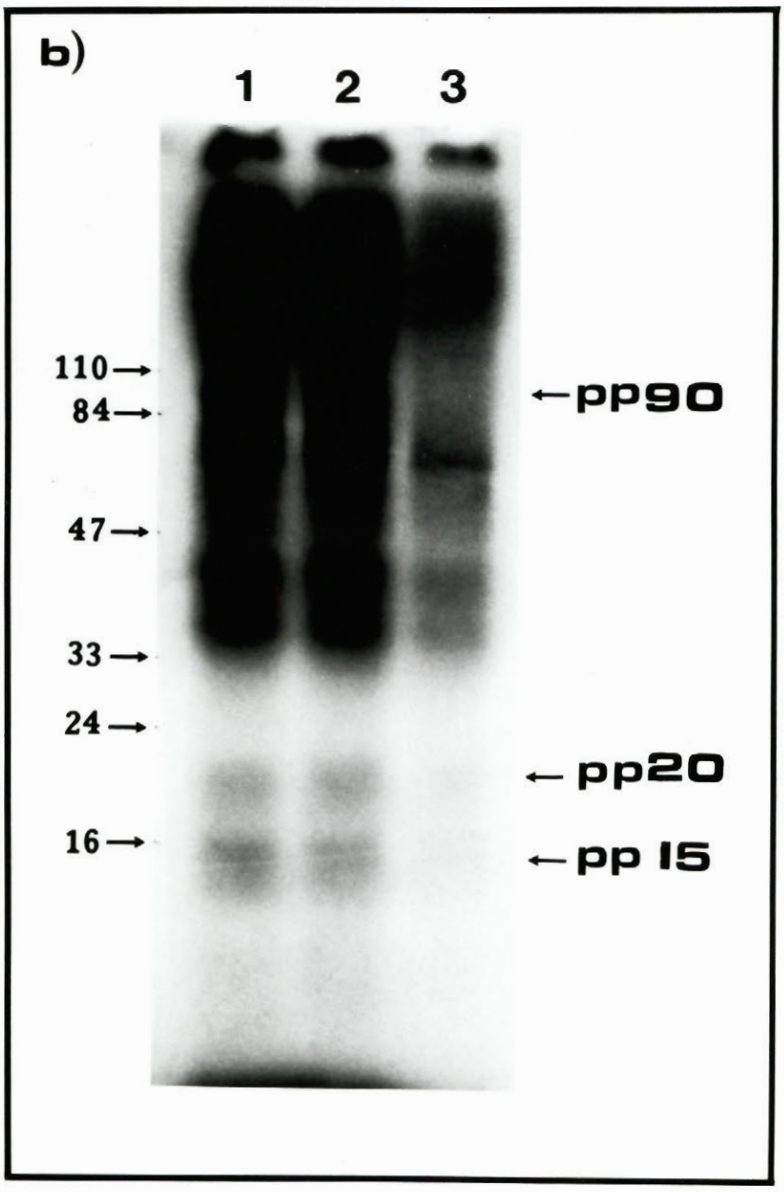

10
1
CPM ratio
Antibody
None
C3C12
MT4
n=8
n=3
n=3
n=4
n=3
E-5
E-5 + Thy
E-5 + Ti-6

**Figure 1b)** SDS-PAGE autoradiography of E-5 cell lysates: lane 1: after 30 min contact with thymocytes; lane 2: after 30 min contact with Ti-6 lymphoma cells; lane 3: cultured alone. Identical amounts of E-5 protein were used in all three lanes. Note that contact with thymocytes and Ti-6 cells induced the neophosphorylation of at leat three proteins, namely pp90, pp20 and pp15.


b)
1
2
3
110
84
47
33
24
16
pp90
pp20
pp 15

When a cellular extract from thymocyte-activated E-5 cells was subjected to SDS-PAGE and autoradiography, we consistently found neophosphorylation of at least three proteins (figure 1b, lane 1). These had molecular weights of 15, 20 and 90 kD respectively and were not phosphorylated in E-5 cells cultured alone for 30 minutes (figure 1b, lane 3). Furthermore, Ti-6 cells, previously shown to interact with E-5 cells through the gp23/45 adhesion molecule (Hugo et al., 1989, and unpublished data], triggered the neophosphorylation of the same three E-5 protein substrates (figure 1b, lane 2). In addition to the above neophosphorylation events, increased phosphorylation of other E-5 protein substrates was also induced by contact with either thymocytes or Ti-6 cells. It must be pointed out that phosphorylation levels of neither thymocytes nor Ti-6 cells were increased by contact with E-5 cells, as determined by total [$^{32}$P]phosphate incorporation and SDS-PAGE analysis (not shown).

## 4.42 Contact-dependence of E-5 protein substrate phosphorylation.

To ascertain that direct lympho-epithelial contact is required for the gp23/45-dependent stimulation of E-5 cells, and to eliminate the possibility that a soluble factor such as a lymphokine or any putative soluble ligand of gp23/45 might trigger these events, we performed an E-5 phosphorylation

experiment using the co-culture system described in section 2.4. When thymocytes or Ti-6 cells were cultured in presence of phosphate-starved E-5 cells, but physically separated from the epithelial cells by a polycarbonate filter, we observed only a 1.12 (thymocytes) and 1.08 (Ti-6) fold increase in total E-5 phosphorylation, compared to a 3.21 (thymocytes) and 2.94 (Ti-6) fold increase when lympho-epithelial cell contact was allowed (figure 2).

**4.43 Tyrosine phosphorylation of gp90.**

Since at least two of the E-5 phosphorylated protein substrates (pp20 and pp90) had molecular weights similar to components of the E-5 adhesion complex (gp23 and gp90), it became important to determine whether any of the phosphorylated substrates were components of the adhesion complex.

The E-5 adhesion complex was immunopurified from lysates of thymocyte-activated (figure 3, lanes 3) and non-activated (figure 3, lanes 1) E-5 cells, using the C3C12 mAb. As a control, an identical amount (in $\mu$g of protein) of thymocyte-activated E-5 cell lysate was "immunoprecipitated" with the MT4 isotype-matched mAb (figure 3, lane 2). SDS-PAGE followed by autoradiography showed that, of the three components of the

**Figure 2:** Necessity of lympho-epithelial contact to induce E-5 cell phosphorylation. Phosphorylation measurments are reported as in figure 1a and represent the increase in total [$^{32}$P]phosphate incorporation per $\mu$g of E-5 protein, obtained by thymocyte or Ti-6 contact with E-5 (horizontal hatch) or by thymocyte or Ti-6 co-culture with E-5 cells, without contact (tengential hatch). In the latter case, the lymphoid cells were seperated from E-5 cells by a polycarbonate filter which prevents lympho-stromal cell contact but allows the free diffusion of soluble proteins. The number of independent observations for each experiment is indicated.

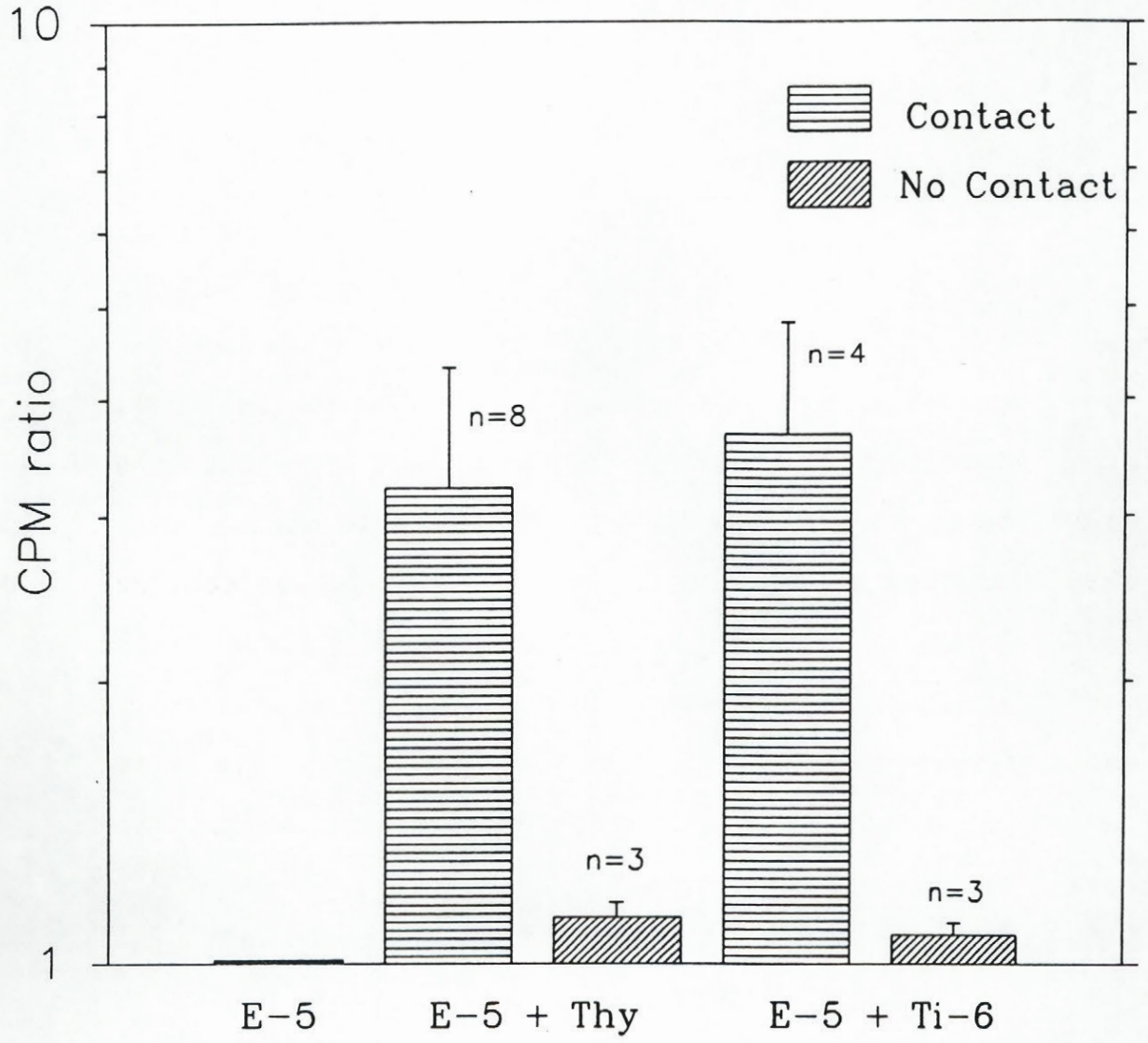
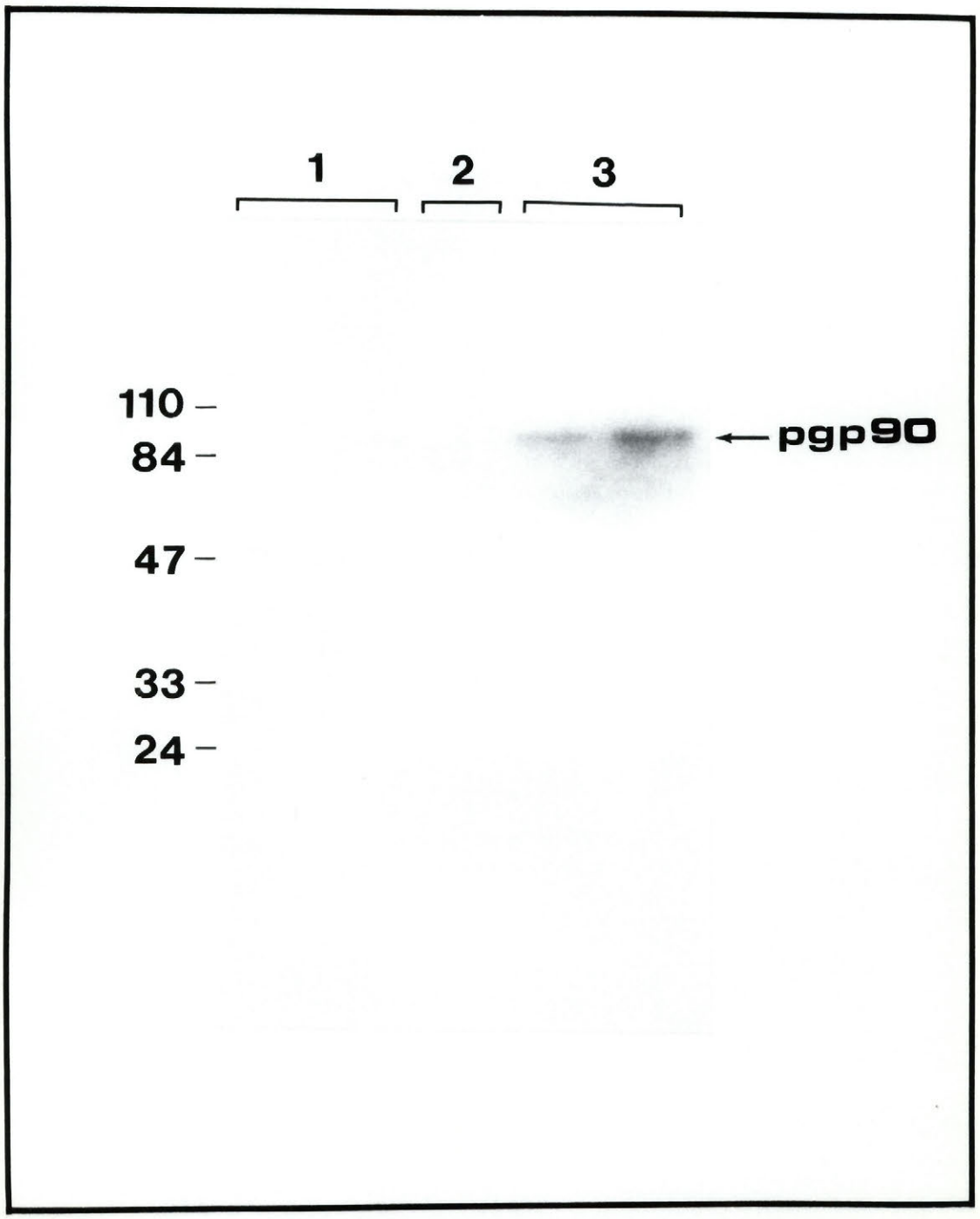

10
1
CPM ratio
Contact
No Contact
n=8
n=3
n=4
n=3
E-5
E-5 + Thy
E-5 + Ti-6

**Figure 3:** Thymocyte contact-dependent phosphorylation of gp90. SDS-PAGE autoradiography of the immunopurified E-5 adhesion complex originating from thymocyte-activated and non-activated E-5 cells. Lane 1: adhesion complex immunopurified by the C3C12 mAb from duplicate lysates of non-activated E-5 cells. Lane 2: immunopurification control: lysate from thymocyte-activated E-5 cells was "immunoprecipitated" using the isotype-matched control mAb MT4. Lane 3: adhesion complex immunopurified by the C3C12 mAb from duplicate lysates of thymocyte-activated E-5 cells. Note that only gp90 from thymocyte-activated E-5 cells (lanes 3) is phosphorylated.


1
2
3
110
84
47
33
24
pgp90

immunopurified adhesion complex, only gp90 was phosphorylated, and only in thymocyte-activated E-5 cell lysates (lanes 3). It is therefore likely that the previously identified pp90 substrate corresponds to the gp90 subunit of the adhesion complex, and that pp15 and pp20 are clearly distinct from any component of the adhesion complex.

To establish which amino acid on gp90 was phosphorylated following a 30 min contact of E-5 cells with thymocytes, the immunopurified adhesion complex was hydrolyzed and subjected to phosphoamino acid thin layer electrophoresis. In the activated complex, tyrosine (Y) was heavily phosphorylated, whereas serine (S) and threonine (T) were not phosphorylated (figure 4). As expected, no phosphorylated amino acids were found in the adhesion complex isolated from non-activated E-5 cells, indicating that tyrosine phosphorylation of gp90 was induced *de novo*. Furthermore, confirmation that gp90 phosphorylation was indeed due to the activity of a protein tyrosine kinase was provided by the addition of the tyrosine kinase inhibitor genistein (10$\mu$g/ml) to a thymocyte/E-5 co-culture. The purified adhesion complex originating from such cultures showed no tyrosine phosphorylation (figure 4).

When the lysate from the above experiment was analysed by SDS-PAGE prior to immunopurification of the E-5 adhesion

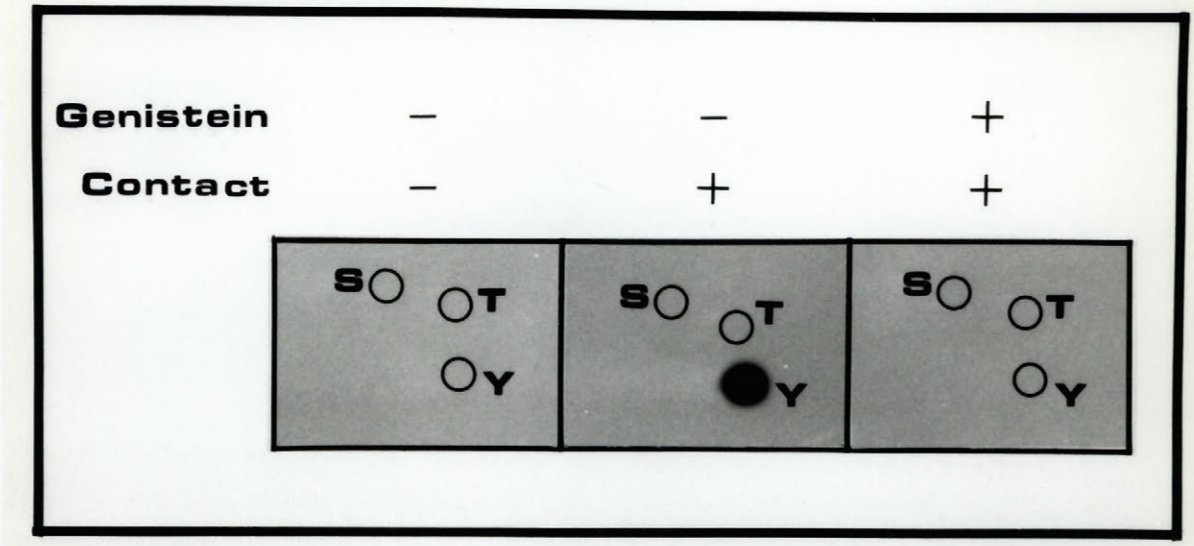


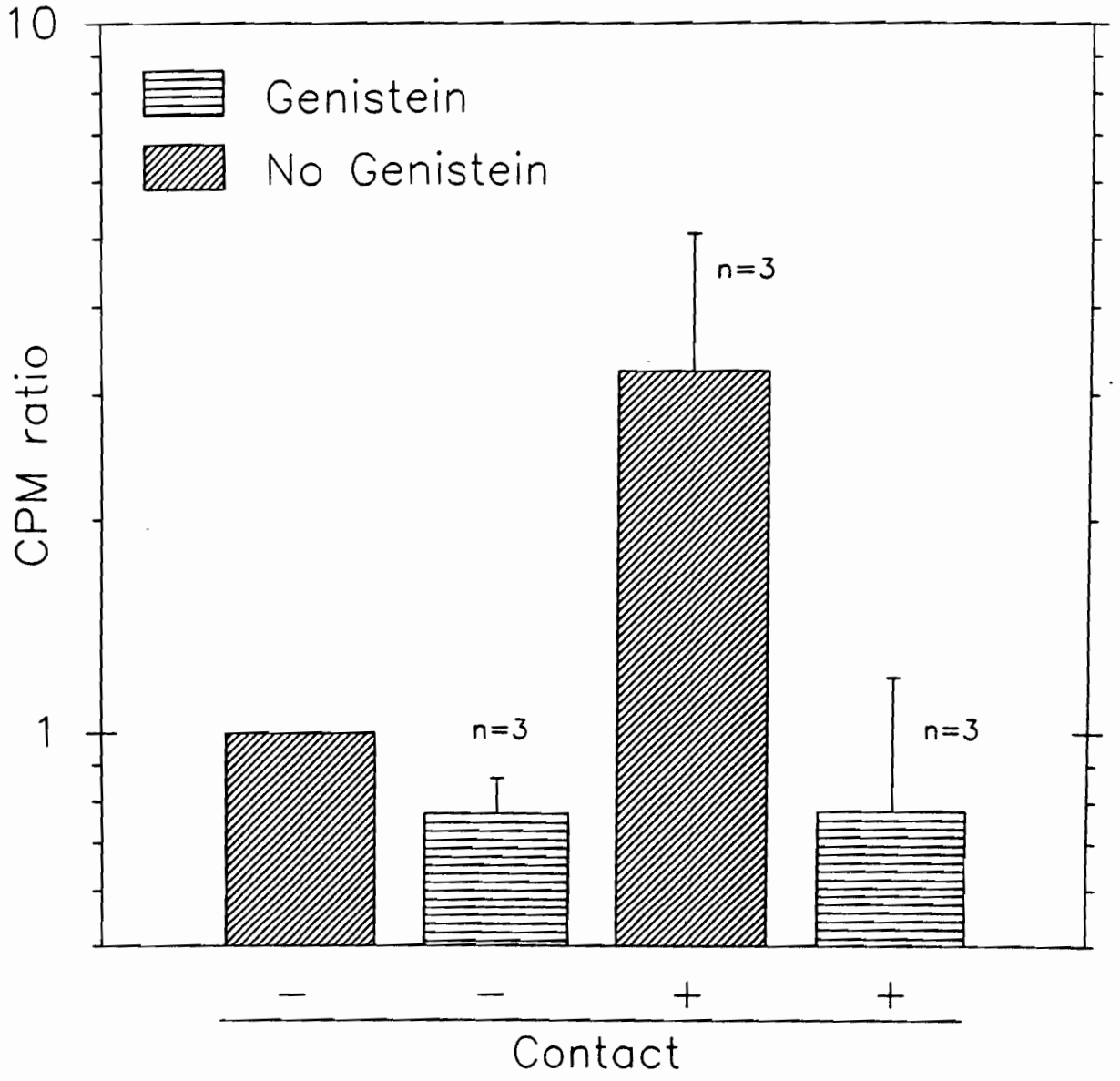

**Figure 4:** Phosphoamino acid analysis of the E-5 adhesion complex immunopurified from non-activated E-5 cells or thymocyte-activated E-5 cells (contact - or +, respectively), in absence or presence of 10 μg/ml genistein (genistein - or +). Note that the tyrosine (Y) phosphorylation of the E-5 adhesion complex is dependent on previous contact with thymocytes, and that this phosphorylation is completely inhibited by genistein. The relative positions of phosphoserine (S) and phosphothreonine (T) are also indicated.

complex, we found that genistein also blocked the thymocyte-induced phosphorylation of p15 and p20 (not shown).

## 4.44 Tyrosine kinase activity associated with the adhesion complex.

The data in section 3.3 demonstrate that the contact-dependent phosphorylation of gp90 (as well as p15 and p20) is mediated by a protein tyrosine kinase. The question as to whether or not this tyrosine kinase activity is an integral part of the adhesion complex was addressed using an *in vitro* immune complex kinase assay (section 2.6). Phosphate-starved E-5 cells and thymocytes were allowed to form complexes for 30 min in absence of [$^{32}$P]phosphate. The E-5 adhesion complex was immunopurified and assayed for its ability to transfer [$^{32}$P]phosphate from exogenous [$\gamma$-$^{32}$P]ATP to gp90, the only protein tyrosine kinase substrate present in the gp23/45/90 complex (figures 3 and 4). [$^{32}$P]phosphate was effectively incorporated by the immunopurified adhesion complex isolated from thymocyte activated E-5 cells (figure 5a), and it was exclusively on the gp90 that phosphorylation was observed when the immune complex was resolved by SDS-PAGE (figure 5b). However, when E-5 cells were not activated by thymocyte contact, or when genistein (10 $\mu$g/ml) was added to the purified adhesion complex of activated E-5 cells, there was no [$^{32}$P]phosphate incorporation and no gp90 phosphorylation. This

**Figure 5:** In vitro kinase activity associated with the E-5 adhesion complex. a) The relative [$^{32}$P]-phosphate incorporation by the immunopurified E-5 adhesion complex originating from E-5 cells stimulated by thymocyte contact (contact +) is compared with that of unstimulated (contact -) E-5 cells (as in previous experiments, the cpm ratio of unstimulated E-5 cells without genistein has a value of 1). Note that when the *in vitro* kinase assay was performed in presence of 10 μg/ml genistein (horizontal hatch), no phosphorylation of the purified gp23/45/90 adhesion complex was observed, even when the adhesion complex was purified from thymocyte-activated E-5 cells.


10
1
CPM ratio
Genistein
No Genistein
n=3
n=3
n=3
−
−
+
+
Contact

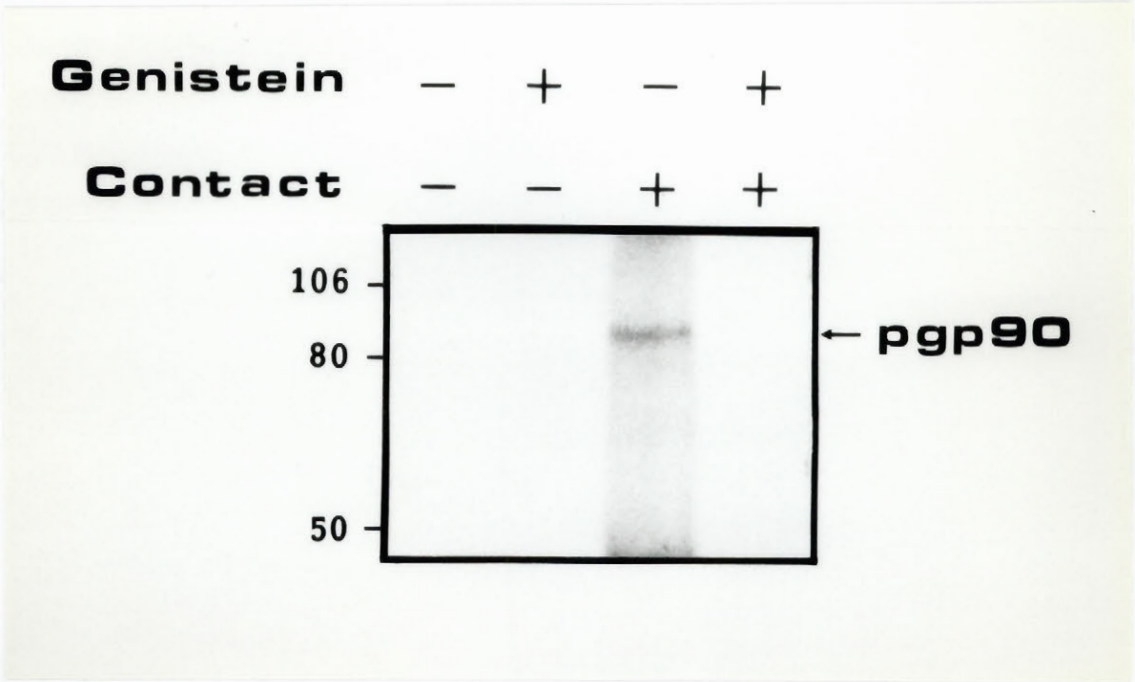



**Figure 5 b)** SDS-PAGE autoradiography of the same preparations as in a) showing phosphorylation of gp90 only in the absence of genistein and after contact with thymocytes.

## 4.5 Discussion

In this work, we report the contact-dependent activation of a thymic epithelial cell line by thymocytes, as determined by increased phosphorylation of epithelial substrates. A number of observations lead us to conclude that lympho-epithelial contact via gp23/45 is both necessary and sufficient to induce epithelial cell phosphorylation. First, the inhibition of gp23/45-mediated interaction by anti-gp23 mAb C3C12 completely abolished the thymocyte-induced phosphorylation of E-5 cells. Second, syngeneic Ti-6 lymphoma cells, also interacting with E-5 cells through the gp23/45 adhesion molecule, triggered the same increase in total [$^{32}$P]phosphate incorporation by E-5 cells (also blocked by C3C12) and the phosphorylation of the same epithelial substrates as thymocytes. Third, thymocytes failed to induce phosphorylation of the gp23$^-$ NG-3 thymic stromal cell line. Taken together, these observations virtually eliminate the possibility that other lympho-epithelial adhesion molecules could be responsible for the induced epithelial phosphorylation. Finally, the use of a co-culture system allowing the two cell types to be cultured in close proximity but seperated by a permeable membrane demonstrated that actual contact between thymocytes and epithelial cells was necessary to induce epithelial cell phosphorylation, thus eliminating the possible participation of soluble factors, such as IL-1 or

γIFN known to activate thymic epithelial cells (Defresne et al., 1990 and Galy et al., 1990), or any other putative cytokine able to interact with gp23/45, such as a soluble form of the thymocyte receptor for the E-5 adhesion molecule.

The most significant finding of the present report was the observation that gp90, non-covalently associated to the gp23/45 adhesion molecule, becomes phosphorylated *de novo* upon thymocyte contact with E-5 cells. Phosphoamino acid analysis revealed that gp90 neophosphorylation is due to the action of a protein tyrosine kinase. This conclusion is corroborated by the observation that genistein is able to block gp90 tyrosine phosphorylation. The remote possibility that the lack of gp90 tyrosine phosphorylation could be due to a toxicity of genistein for E-5 cells was eliminated by a viability test on E-5 cells treated with up to 50 μg/ml of genistein. Because gp90 is physically associated with the gp23/45 adhesion molecule and because its phosphorylation was shown to be gp23/45-dependent, it was logical to ask whether tyrosine kinase activity could be present within the adhesion complex itself. The *in vitro* immune complex kinase assay performed on the purified adhesion complex isolated from activated E-5 cells, revealed the presence of kinase activity within the adhesion complex itself, resulting in the phosphorylation of gp90. Since this phosphorylation is completely blocked by genistein, we conclude that the protein kinase present within

the adhesion complex is in fact a protein tyrosine kinase. Furthermore, because adhesion to thymocytes requires the joint participation of gp23 and gp45 subunits, but not of gp90, the latter is, to our way of thinking, the most likely candidate for the protein tyrosine kinase. An analogous system, with one subunit responsible for binding and the other for signal transduction, has been described with respect to the insulin receptor (Kasuga et al., 1982). If such is the case in our system, gp90 phosphorylation may take place via autophosphorylation. However, since it is possible to immunopurify gp90 only by virtue of its association with gp23 and gp45 (pending the development of a gp90-specific mAb), we cannot formally eliminate the possibility that either gp23 or gp45 is responsible for the phosphorylation of gp90. Furthermore, since gp23/45-mediated contact is also required to trigger the phosphorylation of pp15 and pp20, the possibility exists that either one of these two proteins might be a substrate for this protein tyrosine kinase. Whatever the case may be, it is clear that thymocyte interaction with thymic epithelial cells via the gp23/45 adhesion molecule activates a protein tyrosine kinase within the adhesion complex, which induces the phosphorylation of gp90. Similar mechanisms have been described in cases such as the receptors for insulin (Kasuga et al., 1982 and Jacobs and Cuatrecasas, 1986) epidermal growth factor (Downward et al., 1984) and PDGF (Williams, 1989), where receptor autophosphorylation on

tyrosine residues was induced by interaction of these membrane-bound receptors with their soluble ligands.

It is worth noting that the C3C12 mAb, interacting with the gp23 subunit of the E-5 adhesion molecule, blocks thymocyte adhesion to E-5 cells without inducing [$^{32}$P]phosphate incorporation. Cross-linking of gp23 with C3C12 and a secondary mAb does not induce tyrosine phosphorylation in E-5 cells either (this report and unpublished observation). This is interpreted as indicating that the induction of E-5 phosphorylation may require the cross-linking of gp23 with gp45, as mediated only by a membrane receptor expressed by thymocytes or Ti-6 cells.

There are several possible roles for the thymocyte-induced gp90 phosphorylation. First, it might result in a modulation of the gp23/45 adhesion molecule or the modification of its avidity for $CD4^+CD8^+$ thymocytes, thus causing their release and allowing their further development. The previously observed release of some adhering thymocytes after 90 minutes of *in vitro* contact with E-5 cells (Hugo and Potworowski, 1989) supports this view. In another system, such an activation-dependent regulation of the avidity of the LFA-1 adhesion molecule for its ligand has been described (Dustin and Springer, 1989). In the case of LFA-1, however, its avidity can be regulated either by TCR or CD2-driven

activation (Van Kooyk et al., 1989) whereas in our case, the adhesion avidity would be regulated by activation through the adhesion complex itself. Second, the signal transmitted to the thymic medullary epithelial cells through the adhesion complex might be important for the control of epithelial proliferation and organization. Such a possibility is strongly supported by the recent observation by Shores et al. (1991) that $TCR^+$ thymocytes are necessary for the proliferation and proper maturation of thymic medullary epithelial cells in SCID mice. Since E-5-adhering thymocytes are $TCR^+$ (Hugo and Potworowski, 1990), it is conceivable that the E-5 adhesion complex might be operative in Shores' SCID mouse model. Furthermore, it has been demonstrated in several murine systems (Kaneshima et al., 1987) that the onset of thymic lymphoma can result, especially in the late pre-leukemic period, in the invasion of the entire thymus by thymic medullary epithelial cells. It is therefore possible that the continuous stimulation of the epithelial cells by a gp23/45-adhering pre-leukemic cells could result in the abnormal proliferation of $gp23/45^+$ epithelial cells. Whether proliferation of E-5 cells can be regulated by adhesion complex-mediated contact with thymocytes or Ti-6, or indeed pre-leukemic cells, remains to be elucidated. A first step towards elucidating the effects on thymocytes of gp23/45-mediated adhesion would be to identify the receptor for the E-5 adhesion complex. In this respect, Ti-6 cells, previously proposed as a useful model for lympho-epithelial interactions

(Hugo et al., 1989) were shown in this report to trigger the same gp23/45-dependent phosphorylation events as thymocytes, thus providing a potentially more homogenous and abundant source of functional receptors for gp23/45 than fresh thymocytes.

Although co-culture of thymocytes with E-5 cells did not result in increased phosphorylation of thymocytes under the experimental conditions used, we believe that their gp23/45-mediated interaction with thymic epithelial cells is likely to trigger signal transduction in thymocytes as well. One possible mechanism would be the contact-dependent activation of a phosphatase; in this case, there would be no increased phosphorylation in thymocytes, which was in fact what we observed. Such a mechanism has been demonstrated recently in the human system, where T cell activation through CD2 resulted in the induction of a serine phosphatase (Samstag et al., 1991). The previous observation that thymocytes, having once undergone adhesion, become refractory to subsequent complex formation with E-5 cells (Hugo and Potworowski, 1989) is in good agreement with the concept that a contact-dependent molecular modification has been induced in thymocytes.

Finally, it is possible that thymocyte-induced activation of medullary thymic epithelial cells results in cytokine production by the latter. This has been well documented in the

human system where the LFA-3 adhesion molecule, the counter-receptor of CD2, has been shown to trigger IL-1 secretion by both thymic epithelial cells (Denning et al., 1988) and macrophages (Webb et al., 1990). Released cytokines might in turn participate in the development of thymocytes and/or the regulation of stromal cell development and activity.

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# CHAPTER 5:

## GENERAL DISCUSSION AND CONCLUSIONS.

The E-5 adhesion molecule has been identified as the novel gp23/45/90 complex. The thymocytes interacting with E-5 cells through this adhesion complex induce the stimulation of the epithelial cells and the phosphorylation of the gp90 subunit. In this chapter, we will discuss the physiological relevence of these observations.

## 5.1 Discussion and conclusions.

Our hypothesis that the interaction between $CD4^+CD8^+$ thymocytes and E-5 cells is mediated by a novel adhesion molecule was verified by the identification and characterization of the E-5 adhesion complex, using the adhesion-blocking mAbs L-43 and C3C12. Both antibodies recognize a 23 kD glycoprotein (gp23), which is physically associated to two other subunits, namely gp45 and gp90, which can be co-immunoprecipitated with gp23. While each of these glycoproteins is individually unable to mediate thymocyte adhesion in a solid-phase adhesion assay, the gp23/45 dimer constitutes an active adhesion molecule with the same specificity as the E-5 cells from which it originates.

The gp23/45 adhesion molecule is structurally and functionally different from all other adhesion molecules known today (section 1.5). This was confirmed recently by Palmer et al (1991), who observed that the adhesion of thymocytes to the TS-9 thymic epithelial cell line (Brown et al., 1991) could be significantly inhibited by the C3C12 mAb, but not by antibodies to $H\text{-}2^d$, CD8, LFA-1/ICAM-1, Mel-14, CD2, CD3, Mac-1, Mac-2 or Thy-1. Like E-5 cells, TS-9 was shown to interact principally with $CD4^+CD8^+$ and a few $CD4^+CD8^-$ thymocytes. Moreover, both adhesion systems are trypsin-sensitive, do not depend on the presence of divalent cations and are effective

at 37°C and 4°C (Palmer et al., 1991). Taken together, these observations strongly suggest that the gp23/45 adhesion molecule is responsible, at least partially, for thymocyte adhesion in both systems. Furthermore, the observation that the adhesion of thymocytes to TS-9 cells is insensitive to PI-PLC treatment strongly suggest that, unlike Thy-1, both gp23 and gp45 are transmembrane glycoproteins.

The role of the gp90 subunit, which is physically associated to the gp23/45 dimer (chapter 3), is not clear. Since its association with gp23/45 does not affect the level of $CD4^+CD8^+$ thymocyte adhesion to immobilized gp23/45 *in vitro*, it is unlikely that gp90 might be involved in thymocyte adhesion *per se*. Furthermore, this is supported by the ability of the anti-gp23 mAb L-43 to block the adhesion of $CD4^+CD8^+$ thymocytes to both gp23/45 and gp23/45/90 (chapter 3, table 1), which suggest that gp90 cannot, by itself or in association with gp45, form an active adhesion molecule.

The finding that thymocyte adhesion to E-5 cells through the gp23/45 adhesion molecule leads to the tyrosine phosphorylation of the gp90 subunit is important. It demonstrates the functional and physical association of a protein tyrosine kinase with a thymic stromal adhesion molecule. In this respect, the gp23/45 adhesion molecule could be compared with CD4 or CD8, which are known to be associated

with the $p56^{lck}$ protein tyrosine kinase (PTK) or the T lymphocyte-specific CD2 adhesion molecule, also associated with $p56^{lck}$ (sections 1.512 and 1.522). Since the PTK activity involved in gp90 phosphorylation was present within the immunopurified gp23/45/90 adhesion complex, it is likely that one of the three subunits of the complex might be a PTK. Because the highly conserved catalytic domains of all PTKs have an average molecular weight of 30 kD (Hanks et al., 1988), the gp23 subunit appears to be an unlikely PTK candidate. Another common feature of PTKs is the regulation of their catalytic activity by autophosphorylation of a regulatory domain often located at the C-terminal tail of the PTK (Ullrich and Schlessinger, 1990). Such autophosphorylation has been observed with the src-related non-receptor PTKs $p56^{lck}$ and $p60^{src}$ (Section 1.5123 and Cantley et al., 1991) as well as with receptor PTKs such as the EGF receptor (Margolis et al., 1989 and Hazan et al., 1990), the PDGF receptor (Kazlauskas and Cooper, 1989) and the insulin receptor (Tornqvist and Avruch, 1988). Since most PTKs have autophosphorylation activity, and since gp90 is the only tyrosine-phosphorylated subunit of the purified gp23/45/90 complex, it appears likely that gp90 itself might be the PTK associated with the E-5 adhesion molecule. We therefore propose a model (figure 1) in which the adhesion of $CD4^{+}CD8^{+}$ thymocytes to E-5 cells via the gp23/45 adhesion molecule leads to the activation of a PTK activity present within the

adhesion complex itself, and which results in the phosphorylation of gp90 and other cellular substrates, such as p15 and p20. The question as to whether or not gp90 is the PTK responsible for these events will remain unanswered until a specific reagent to gp90 is developed. However, given the recently recognized importance of tyrosine phosphorylation in the regulation of integrin-mediated cell adhesion (Pullman and Bodmer, 1992), the functional characterization of gp90 must be a priority.

The adhesion of $CD4^+CD8^+$ thymocytes to medullary epithelial cells might be viewed as a paradox in the light of our present understanding of intrathymic T cell differentiation. Indeed, double positive thymocytes are found almost exclusively in the cortical region of the thymus, while the gp23/45 adhesion molecule is expressed only in the medulla, as demonstrated by the reactivity of L-43 and C3C12 on frozen thymic sections. This raises questions about the *in vivo* relevance of these observations. Can $CD4^+CD8^+$ thymocytes interact with medullary epithelial cells *in vivo*? Based on previous observations from our laboratory, the answer to this question is probably yes. It was indeed shown, using a monoclonal antibody capable blocking the adhesion of thymocytes to E-5 cells, that the E-5 adhesion molecule could be located *in situ* at the contact sites between thymocytes and thymic medullary epithelial cells (Zelechowska et al., 1986).

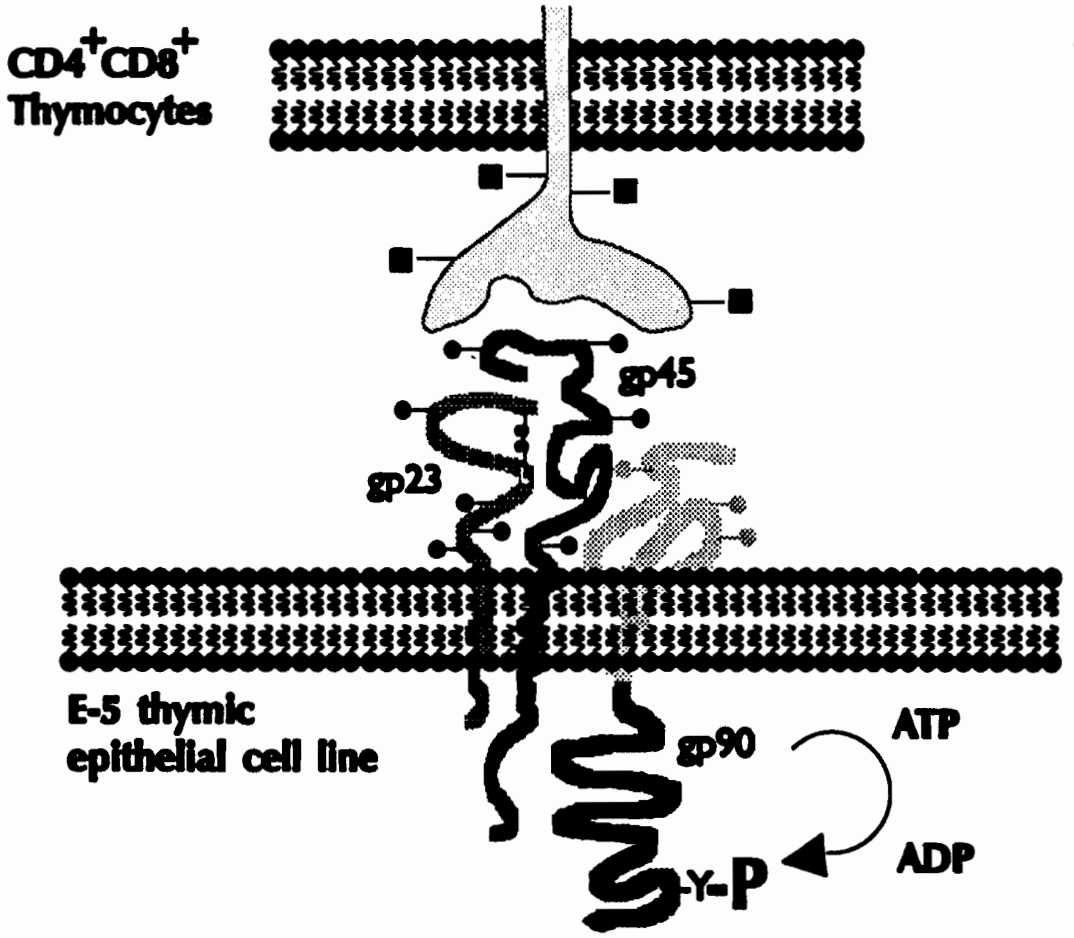


**Figure 1:** The gp23/45/90 thymic epithelial adhesion complex. Both the gp23 and gp45 subunits are necessary for thymocyte adhesion. A putative thymocyte receptor for gp23/45 is represented, but its structure is unknown. The interaction of thymocytes with E-5 through the gp23/45 adhesion molecule leads to the phosphorylation of the gp90 subunit, which may be a PTK with autophosphorylation activity. At this point, the transmembrane and extracellular domains of gp90 can only be postulated

This suggests that the gp23/45$^+$ medullary epithelial cells located closer to the cortico-medullary junction could interact with CD4$^+$CD8$^+$ thymocytes. Furthermore, the few CD4$^+$CD8$^-$ thymocytes found to adhere to E-5 cells (Hugo and Potworowski, 1990) and to the purified gp23/45 complex might be another thymocyte population interacting with gp23/45$^+$ epithelial cells *in situ*. However, since CD4$^+$CD8$^-$ thymocytes can be found in the medulla (where they express the TCR/CD3 complex) and in the cortex (where they are CD3$^-$), a further characterization of the few adhering CD4$^+$CD8$^-$ thymocytes will be necessary.

The provocative observation that interactions between CD4$^+$CD8$^+$ thymocytes and thymic medullary epithelial cells can result in signal transduction in the latter raises questions about the role of such interaction *in situ*. Do we have any evidence that thymocyte adhesion can control the activity or the function of thymic stromal cells ? Recently, it was reported that murine thymic medullary epithelial cell lines can produce IL-1 (Farr et al., 1989). In the human system, it was shown that thymocyte adhesion to thymic epithelial cells through the CD2/LFA-3 pathway (see section 1.522) can trigger the contact-dependent production of IL-1 by the stromal cells (Le et al., 1990). Furthermore, preliminary results from our own laboratory indicate that E-5 cells can be stimulated by thymocytes to produce IL-1 and other unidentified cytokines,

in a contact-dependent manner. This IL-1, produced by thymocyte-stimulated medullary epithelial cells might induce the production of IL-2 by $CD4^-CD8^-CD3^-$ thymocytes (Rothenberg et al., 1990) as well as the expression of the high affinity IL-2 receptor by these thymocytes (Falk et al., 1989). The resulting autocrine stimulation of these $CD4^-CD8^-$ thymocytes is postulated to induce their proliferation and the expression of the TCR (Jenkinson et al., 1987). These considerations lead us to postulate that the stimulation of thymic medullary epithelial cells by $CD4^+CD8^+$ thymocytes might be involved in the regulation of the development of the more immature $CD4^-CD8^-$ thymocytes. The question of the participation of gp23/45-mediated interactions in the development of T lymphocytes in the thymus has been addressed by blocking such interactions using anti-gp23 monoclonal antibodies. The injection of C3C12 mAb to newborn mice (unpublished experiments from our laboratory), as well as the *in vitro* treatment of fetal thymic organ cultures with C3C12 (FTOC experiments performed in the laboratory of Dr. W. van Ewijk) had no significant effect on intrathymic thymocyte differentiation (unpublished). These observations, although negative, support our hypothesis that gp23/45-mediated interactions are not involved in the differentiation of adhering thymocytes. However, the participation of $gp23/45^+$ epithelial cells in the development of non-adhering thymocytes, via the secretion of cytokines such as IL-1 for example, cannot be ruled out. Even if the

gp23/45-dependent secretion of IL-1 is blocked by C3C12, IL-1 might still be produced by other thymic stromal cells, through other adhesion pathways, such as CD2/LFA-3.

Lympho-stromal interactions in the thymus might also play a role in the proper development and organization of the thymic stromal cells themselves. This view is based on a recent report by Shores et al. (1991), who showed that the absence of medullary epithelial cells in the thymus of SCID mice might be due to the total absence of $TCR^+$ thymocytes resulting from this genetic defect. Indeed, the introduction of normal bone marrow precursor cells and, hence, of normal TCR+ thymocytes into these mice led to the appearance of medullary epithelial cells and to the restoration of the thymus anatomy. The authors did not address the possibility that cytokines produced by these $TCR^+$ thymocytes, or that the TCR itself, might be responsible for these events. However, the fact that the earliest $TCR^+$ thymocytes have the same phenotype as gp23/45-adhering $CD4^+CD8^+$ thymocytes, and that gp23/45-mediated interactions generate a signal in the epithelial cells might suggest that lympho-stromal interactions are required.

The experiments presented in this thesis allowed us not only to identify a novel thymic adhesion molecule, but also to clearly demonstrate that lympho-stromal interactions can

result in the stimulation of the stromal cells involved. Although the interaction may not trigger a direct differentiation signal for the adhered thymocytes, it might nonetheless be vital for the correct development of both T lymphocytes and thymic stromal elements. The thymus must therefore be viewed as an organ where the functional homeostasis depends on the symbiotic relationship between thymocytes and stromal cells.

## 5.2 References.

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# CLAIMS TO ORIGINALITY

Chapter 2:

A solid-phase adhesion assay has been developed. It allows the immobilization of membrane glycoproteins in a functional configuration. Although this method was originally designed for the study of T cell activation by immobilized antigens, we present the first experimental evidence that such assay can be used for the functional identification of complex adhesion molecules.

Chapter 3:

This chapter is the first description of the E-5 adhesion molecule, the gp23/45/90 adhesion complex. The purification of each subunit by immunoprecipitation, electrophoresis, electro-elution and renaturation allowed us, using the solid-phase adhesion assay, to determine that gp23 and gp45 were sufficient for thymocyte adhesion. Furthermore, we were able to demonstrate that the purified gp23/45 adhesion molecule had the same binding specificity as the E-5 cells from which it was isolated. The structure, properties and specificity of this adhesion molecule are unique.

Chapter 4:

This chapter demonstrates that thymocyte adhesion via the gp23/45 adhesion molecule induces the phosphorylation of the gp90 subunit on tyrosine residues. It is therefore the first evidence that thymocytes can induce a tyrosine kinase signal transduction pathway in thymic stromal cells. Furthermore, we have shown that the protein kinase induced by gp23/45-mediated contact was physically associated to the E-5 adhesion molecule. These observations open the way for a further investigation of gp23/45/90 adhesion complex involvement in the regulation of thymic stromal cell activity by $CD4^+CD8^+$ thymocytes.

# APPENDIX I

## PUBLICATIONS

1. Potworowski, E.F., Hugo, P. and Couture C.: Binding of cortical thymocytes to a medullary epithelial cell line: A brief review. Thymus **13**:237-243 (1989).

2. Couture C., Patel, P.C. and Potworowski, E.F.: A novel thymic epithelial adhesion molecule. Eur. J. Immunol. **20**:2769-2773 (1990)

3. Couture C., Amarante-Mendes G. and Potworowski, E.F.: Tyrosine kinase activation in thymic epithelial cells: necessity of thymocyte contact through the gp23/45/90 adhesion complex. Eur. J. Immunol. (in press).

4. Couture C. and Potworowski, E.F. The thymus: a complex network of intercellular signalling. Médecine/Science 8:572

## COMMUNICATIONS

1. Hugo, P., Couture, C. and Potworowski, E.F.: Lympho-stromal complex formation in the thymus: who does what to whom ? 2nd workshop on thymus histophysiology, Rolduc, The Netherlands, April 2-6 1989.

2. Couture C. and Potworowski, E.F.: Identification of two thymic epithelial glycoproteins specifically bound by thymocytes. J. Cell. Biochem. **14A**:187. 1990. UCLA Symposia on Molecular and Cellular Biology: Molecular Basis of Cellular Adhesion. Steamboat Springs, Colorado, USA, January 20-26, 1990.

3. Couture, C., Patel, P.C. and Potworowski, E.F.: Thymic lympho-stromal complexes: Identification of a novel adhesion molecule. Canadian Society for Immunology, 4th Spring Meeting, Mont Gabriel, Québec, Canada, March 9-12,1990.

4. Couture, C., Amarante-Mendes, J.G.P., Patel, P.C. and Potworowski, E.F.: Impairment of T cell development by in vivo blocking of the gp23/45 thymic epithelial adhesion molecule. Canadian Society for Immunology, 5th Spring meeting, Lake Louise, Alberta, Canada, March 8-12, 1991.

5. Potworowski, E.F., Couture, C. and Amarante-Mendes, G.: The thymic medullary adhesion molecule. The thymus workshop, Rolduc, The Netherlands, April 7-11, 1991.

6. Couture, C., Amarante-Mendes, G. and Potworowski, E.F. Thymocyte contact induces protein tyrosine kinase activation in thymic epithelial cells. Canadian Society

for Immunology, 6th spring meeting, Mont Gabriel, Qc, Canada, march 13-16, 1992.

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8. Potworowski, E.F., Couture, C., Hébert, B. and Beauchemin, C: Activation of thymic medullary epithelial cells by contact with thymocytes. The thymus workshop, Rolduc, The Netherlands, April 26-29, 1992.