CONTROL OF GENE EXPRESSION AND CELL GROWTH BY THE NF-xB/IxB FAMILY OF TRANSCRIPTION REGULATORS

by

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A Thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Abstract

The NF- κ B/Rel family of transcription factors participates in the activation of genes involved in immune response, growth control and development. NF-kB/Rel is retained in the cytoplasm by inhibitory molecules, collectively termed IxB. Cell activation leads to the phosphorylation, ubiquitination and proteasome mediated degradation of $I\kappa B\alpha$, thus permitting NF-xB/Rel translocation to the nucleus and target gene activation. The objective of this research was to understand the mechanisms by which $I\kappa B\alpha$ regulates NF- κ B/Rel activity and modulates coll growth. Overexpression of I κ B α antisense RNA in NIH 3T3 cells led to decreased $I\kappa B\alpha$ protein levels, increased NF- κB dependenttranscription and induced malignant transformation. In contrast, overexpression of $I\kappa B\alpha$ negatively affected cell growth and NF- κ B activity, suggesting that I κ B α represents a potential growth suppressor activity. To characterize the signaling events contributing to IkB α phosphorylation and degradation, an IkB α -associated kinase was isolated and characterized as casein kinase II (CKII). Mutation of three residues phosphorylated by CKII in vitro (S283A; T291A; T299A) abolished constitutive phosphorylation of IxB α in vivo. The C-terminal CKII sites were not required for inducible $I\kappa B\alpha$ degradation, but rather contributed to intrinsic protein stability. Deletion of a region located between aa 269 to as 287 abolished the ability of $I\kappa B\alpha$ to inhibit NF- κ B-dependent transcription. dramatically reduced IkBa intrinsic stability and rendered IkBa insensitive to inducermediated degradation. Proteasome inhibitors and mutation of N-terminal serines (S32A: S36A) blocked inducer-mediated degradation of $I\kappa B\alpha$ and interestingly, stabilized the turnover of C-terminal deletion mutants. Thus, the C-terminus appears to protect IxBa from the constitutive activity of the proteasome. IxB α molecules mutated in N-terminal serine residues (S32A: S36A) efficiently blocked human immunodeficiency virus (HIV-1) long terminal repeat transactivation and multiplication. Strikingly, the C-terminal CKII sites were also required for this inhibitory function. These experiments suggest a strategy that may contribute to the inhibition of HIV-1 gene expression by interfering with NF-kB/Rel signaling pathway.

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Résumé

La famille des facteurs de transcriptions NF-xB/Rel participe à l'activation de gènes impliqués dans la réponse immune, le contrôle de la croissance cellulaire et le développement embryonaire. NF-xB est retenu dans le cytoplasme par des molécules inhibitrices, collectivement appelées IxB. L'activation cellulaire induit leur phosphorylation, ubiquitination et dégradation par le protéasome, et de ce fait permet à NF- κ B/Rel d'être mobilisé vers le noyau et d'activer les gènes cibles. Le but de cette recherche est de comprendre les mécanismes par lesquels IxBo contrôle l'activité NF- κ B/Rel et module la croissance cellulaire. La surexpression d'ARN I κ B α antisense dans les cellules NIH 3T3 entraîne une diminution des niveaux d'expression d'I κ B α , une augmentation de la transcription dépendante de NF- κ B et la transformation cellulaire. Par contre, la surexpression d'Ik $B\alpha$ a un effet négatif sur la croissance cellulaire et l'activité NF-kB, ce qui suggère qu'lkBa représente potentiellement une activité inhibitrice de la croissance cellulaire. Afin de caractériser les signaux induisant la phosphorylation et la dégradation d'I κ B α , une kinase associée à I κ B α a été isolée et identifiée comme étant la caséine kinase II (CKII). La mutation de trois résidus phosphorylés par CKII in vitro (S283; T291;T299) abolie la phosphorylation constitutive d'IxBa in vivo. Les sites CKII C-terminaux ne sont pas requis pour la dégradation induite d'IkBa, mais contribuent par contre à la stabilité intrinsèque de la protéine. La délétion de la région située entre les as 269 à 287 abolie la capacité d'I κ B α d'inhiber la transcription dépendante de NF-kB, réduit de façon importante la stabilité intrinsèque d'IxB α et protège IxB α de la dégradation inductible. Les inhibiteurs du protéasome, de même que la mutation des sérines N-terminales (S32:S36) empêchent la dégradation d'IxBa induite par stimulation et fait intéressant, stabilisent les mutants tronqués en position C-terminale. Par conséquent, le C-terminus semble protéger IkBa de la dégradation constitutive par le protéasome. Les molécules d'IxBa dont les sérines

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N-terminales ont été substituées (S32A; S36A), inhibent efficacement la *trans*activation de la longue séquence répétée terminale et la multiplication du virus de l'immunodéficience humaine (VIH-1). Fait intéressant, les sites CKII C-terminaux sont essentiels pour l'inhibition de la multiplication virale. Ces résultats suggèrent une stratégie pouvant contribuer à l'inhibition de l'expression du VIH-1 en interférant avec les signaux de transduction de la voie NF- κ B/Rel.

Acknowledgments

I express my deepest gratitude to my supervisor Dr. John Hiscott for his guidance and support during the course of my Ph.D. study. His enthusiasm together with the time and effort he invested in the supervision of my work has made this experience a true success.

The mutagenesis of $I \times B \alpha$ was a very intense research field and without the collaboration of Dr. Rongtuan Lin, the competition would have left us far behind. Dr. Lin had the wisdom to stay calm and efficient even when we knew that other groups were trying to publish related results. Working with Dr. Lin was a real pleasure and I will always remember him as the man with the "Golden Hands".

I would like to thank my friend and collaborator Anne Roulston for her support at the beginning of my training and for generating our collection of NF-kB antibodies. These antibodies have been essential tools in the course of my research. I also would like to express my gratitude to Ivy Kwan, Richard Bitar, Peter Chou, Hakju Kwon, Michelle Clarke, Normand Pépin and Evgenia Garoufalis for their participation at different stages of my work. I also wish to acknowledge the support and friendship from the members of the laboratory. In particular I would like to thank Raymond Lee, Pascale Crepieux, Hannah Nguyen, Louisa Petropoulos and Carmela Deluca for suggestion on this document.

I am grateful to the Medical Research Council of Canada for supporting me financially during the course of my Ph.D. study. I thank Drs. M. Gossen and H. Bujard for the tetracycline responsive plasmids and helpful information about selection of cells, as well as Dr. A. Cochrane for helpful discussions. I am grateful to MyoGenics Inc. for supplying the peptidyl aldehyde MG132 and to the AIDS Research and Reference Program for supplying plasmids and antisera. I would like to thank Dr. Antonis Koromilas and Dr. M. Alaoui-Jamali for critical reading and helpful comments on manuscripts.

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Preface

In accordance with the Guidelines for Thesis Preparation, the candidate has chosen to present the results of his research in classical form. A general introduction is presented in chapter I and appears in part in the following review article:

 Beauparlant, P. and Hiscott, J. 1996. Biological and biochemical inhibitors of the NF-κB/Rel proteins and cytokine synthesis. Cyt.& Growth Fact.Rev. *In Press*.

The materials and methods used in this thesis are presented in chapter II. The results are described in chapters III to VI and appear in part in the following articles, in the same order:

- Beauparlant, P., Kwan, I., Bitar, R., Chou, P., Koromilas, A.E., Sonenberg, N. and Hiscott, J. 1994. Disruption of IκBα regulation by antisense RNA expression leads to malignant transformation. Oncogene 9:3189-3197.
- Lin, R., Beauparlant, P., Makris, C., Meloche, S. and Hiscott, J. 1996. Phosphorylation of IκBα in the C-terminal PEST domain by casein kinase II affects intrinsic protein stability. Mol.Cell.Biol. 16:1401-1409.
- 4. Beauparlant, P., Lin, R. and Hiscott, J. 1996. The role of the C-terminal domain of IκBα in protein degradation and stability. J.Biol.Chem. 271:10690-10696.
- Beauparlant, P., Kwon, H., Clarke, M., Lin, R., Sonenberg, N., Wainberg, M. and Hiscott, J. 1996. Transdominant mutants of IκBα block Tat-TNF synergistic activation of HIV-1 expression and virus multiplication. J.Virol. 70:10690-10696.

The work presented in chapter III to VI was part of a very active project shared by other researchers in the laboratory. Therefore, the results obtained by Dr. Rongtuan Lin,

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Constantin Makris and Dr. Sylvain Meloche, published in reference no. 3, are summarized at the beginning of chapter IV to introduce the work performed by the candidate. Dr. Rongtuan Lin was also responsible for the results obtained in figures 21 and 22. Results obtained by Hakju Kwon and Michelle Clarke were important elements in the rationale of chapter VI and are presented in figures 36, 37 and 39. The candidate also wishes to acknowledge the excellent technical assistance of Ivy Kwan, Peter Chou and Richard Bitar for the work published in chapter III. References cited in this thesis are listed in chapter VIII.

The candidate was also involved in collaborations with other researchers in the laboratory which resulted in the following publications:

- Roulston, A., Beauparlant, P., Rice, N.R. and Hiscott, J. 1993. Chronic human immunodeficiency virus type 1 infection stimulates distinct NF-kB/rel DNA binding activities in myelomonoblastic cells. J.Virol. 67:5235-5246.
- 7. Bitar, R., Beauparlant, P., Lin, R., Pitha, P. and Hiscott, J. 1995. Retrovirusmediated transfer of nuclear factor- κ B subunit genes modulates I κ B α and interferon β expression. Cell Growth & Differ. 6:965-976.
- Roulston, A., Lin, R., Beauparlant, P., Wainberg, M.A. and Hiscott, J. 1995. Regulation of HIV-1 and cytokine gene expression in myeloid cells by NFxB/Rel transcription factors. Microbiol.Rev. 59:481-505.

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Chapter I

General Introduction

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1. NF-kB/Rel transcription factors

The NF- κ B/Rel family of transcription factors participates in the activation of a diverse range of genes involved in inflammation, immunological responsiveness, lymphoid differentiation, growth control, and dorsal-ventral axis determination in Drosophila (reviewed in 12, 238, 281 and 288). NF-kB exists in the cytoplasm in latent form as a complex consisting of a dimer of DNA-binding subunits bound to an inhibitor IxB. Activating agents, such as virus infection, cytokines (including TNF- α and IL-1), lipopolysaccharide (LPS) and phorbol esters induce the degradation of cytosolic $I_{\kappa}B\alpha$. thereby promoting release and nuclear translocation of NF-KB/Rel dimers (12.238.281.288). NF- κ B-regulated genes encode proteins involved in the rapid response to pathogens or stress, including cytokines (IFN- β , TNF- α , IL-1, IL-2, IL-6, IL-8 and chemotactic proteins), cytokine receptors, cellular adhesion molecules (ELAM-1, VCAM-1, ICAM-1 and E-selectin), hematopoietic growth factors (M-CSF, G-CSF and GM-CSF) and acute phase reactants. The microfilament protein vimentin and inducible nitric oxide synthase (iNOS) are among many other genes controlled by NF-kB. Interestingly, the early gene expression of several viruses including the human immunodeficiency virus-1 (HIV-1), cytomegalovirus and adenovirus is also regulated by NF- κ B and thus these viruses utilize this aspect of the immune response to their selective replicative advantage (12,238,281,288).

This introduction will cover four aspects of NF- κ B/I κ B biology: 1) an overview of the NF- κ B/I κ B pathway; 2) an analysis of the biological and biochemical inhibitors used to interfere with NF- κ B activation; 3) the role of the NF- κ B/rel factors in cell growth control and oncogenesis; and 4) the role of NF- κ B in HIV-1 transcription regulation. Given the pleiotropic role of NF- κ B transcription factors in controlling genes involved in the inflammatory response, immunoregulation and stress response, the use of inhibitory compounds promises to play an increasingly important role in suppressing pathological inflammatory responses, including toxic/septic shock, graft-vs-host reactions, chronic inflammatory conditions and radiation injury (12). The inhibition of NF- κ B activation by steroid hormones, antioxidants, specific protease inhibitors and other compounds may provide a pharmacological basis for interfering with many pathologies including cancer, AIDS and infectious diseases (12).

1.1 NF-kB/Rel DNA binding subunits.

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Several excellent reviews have recently covered the NF- κ B/Rel pathway in detail (12,238,281,288). Each of the five DNA-binding members of the NF-xB/Rel family (p50, p52, ReIA, c-Rel and RelB) shares a region of homology termed the Rel homology domain (RHD) which spans approximately 300 amino acids at the amino terminus of each protein (Figure 1). The N-terminal half of the RHD contains a motif (Arg-X-X-Arg-X-Arg-X-Cys) that is conserved in all NF- κ B/Rel proteins and is essential for DNA binding (151,283). The C-terminal portion of the RHD contains the region responsible for dimerization of NF-xB/Rel proteins (41,175). A conserved cluster of positively charged amino acids (Arg-Lys-Arg-Gln-Lys and Lys-Arg-Lys-Arg) which have been shown to act as a nuclear localization signal (NLS) in other nuclear proteins (33,95,102,103) is also present in the C-terminal half of the RHD. The NLS is essential for the translocation of NF-kB/Rel proteins to the nucleus and for NF-kB/Rel proteins activity (196,197). The crystal structure of p50 homodimers bound to DNA reveals that each DNA-binding subunit surrounds DNA by folding into a pattern that resembles immunoglobulin-like modules (100,201). The RHD DNA-binding region is divided into two domains separated by a flexible hinge: the amino-terminal portion contains a recognition loop which interacts within the major groove of DNA and the carboxyterminal region functions as a dimerization interface (100,201).

Two of the DNA-binding subunits are synthesized as inactive cytoplasmic precursor proteins. The product of the *nfkb1* gene is a 105 kDa protein which gives rise to a 50 kDa DNA-binding subunit, p50 by proteolytic cleavage (Figure 1). The *nfkb2* encoded

Figure 1. Functional domains of NF- κ B proteins. All NF- κ B proteins share the Rel homology domain (RHD) (cross-hatched pattern). The RHD mediates DNAbinding, dimerization and nuclear localization functions. The NF κ B1/p105 and NF κ B2/p100 precursor proteins have a long C-terminal domains containing seven ankyrin repeats (solid boxes). The C-terminal domain is proteolytically cleaved at a site (black triangle) located near a flexible glycine-rich hinge (horizontal line pattern) to generate the transcription factors NF κ B1/p50 and NF κ B2/p52. RelA and c-Rel in contrast contain transactivation domains in the C-terminal part of the protein (vertical line pattern).

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p100 precursor is similar to p105 with a mature DNA-binding form of p52. likewise generated by limited proteolysis. Each precursor protein has two functional domains: the carboxy terminal inhibitory domain containing seven ankyrin repeats and the amino terminal RHD. A glycine rich region divides the two functional domains and allows sufficient structural flexibility for amino and carboxy termini to physically interact (117). This physical interaction masks the nuclear localization sequence within the RHD, preventing nuclear translocation and DNA binding. The processed DNA binding subunits which are composed almost entirely of the RHD, arise following ubiquitin dependent proteolytic degradation of the carboxy termini up to a site adjacent to the glycine rich hinge (82.213). The other NF- κ B/Rel DNA-binding proteins are not proteolytically processed from precursors but remain cytoplasmic through interactions with inhibitory molecules. c-Rel and RelA(p65) are highly related and in addition to the RHD, they contain an acidic carboxy terminal domain with strong transactivation potential (46.130.218.254-256). Finally, the RelB subunit contains an elongated amino terminal segment but lacks transactivating potential (241).

Once NF- κ B DNA-binding activity is activated, numerous target genes are selectively regulated by the transcriptional activation potential of different homo- and heterodimer combinations. As well, variations in the NF- κ B consensus sequence (5'-GGGANNYYCC-3') to which the subunits bind (153) and cooperativity between different transcription factor families and NF- κ B/Rel contribute to the differential specificity of gene activation (166,218).

The physiological role of the NF- κ B DNA binding subunits has been characterized in mice homozygously deficient in individual NF- κ B proteins. RelA is important in embryogenesis and RelA-/- embryos resorb late in gestation with liver degeneration resulting from extensive hepatocyte specific apoptosis. Nonetheless, hematopoietic cells are formed normally in the absence of RelA. GM-CSF and I κ B α transcriptional activation by TNF- α stimulation is compromised in RelA deficient fibroblasts, illustrating the crucial role of ReIA in inducer mediated NF- κ B transcription (26).

Mice deficient in NF κ B1 (p105/p50) develop normally but suffer from immunological defects (263). In particular, p105/p50-/- B cells are less responsive to anti-Ig antibodies and are not responsive to LPS induced proliferation and NF- κ B activation. Illustrating the complexity of NF- κ B dependent gene regulation, the induction of IFN- β transcription by Sendai Virus infection is augmented in p105/p50-/fibroblasts relative to wild type cells. NF- κ B1/p50 homodimers lack transactivation activity and within the context of the IFN- β promoter. p50 acts as a repressor (32). As a result of increased IFN- β production, p50-/- mice are more resistant to ECM virus infection. Finally, p105/p50-/- macrophages secrete less IL-6 upon LPS stimulation, whereas secretion of IL-1 and TNF- α is unaffected in these animals(263).

Expression of c-Rel is largely restricted to hematopoletic organs and the highest levels of expression are found in B and T cells. Mice lacking c-Rel have a phenotype resembling mice lacking NFxB1 (p105/p50). They undergo normal embryonic development and hematopoletic development is not compromised. However, as with mice lacking p105/p50, Ig production is reduced following antigenic challenge and c-Rel-/- B cells are refractory to LPS and Ig cross-linking stimulation. Anti-CD3 antibodies also fail to stimulate T cell proliferation in c-Rel-/- mice and IL-2 production is reduced compared to wild type T cells. T cell proliferation induced by anti-CD3 antibodies is rescued by the addition of exogenous IL-2, indicating that inhibition of cytokine secretion is responsible at least in part for the immune defect (155).

RelB expression is mainly restricted to dendritic cells of the thymic medulla and secondary lymphoid organs. Mice deficient in RelB develop normally but suffer from a severe inflammatory syndrome with mixed granulocyte/monocyte infiltration in various organs and hypotrophy of the thymic medulla. These mice are deficient in dendritic cells indicating that RelB plays a critical role in their survival or differentiation.

Irradiated spleen cells from RelB-/- mice are poor allogenic stimulators of T cell proliferation as a result of a deficiency in antigen presenting cells. Moreover, these mice are impaired for delayed type hypersensitivity. Nonetheless, expression of TNF- α , IL-1, IL-2 and IFN- γ is not compromised in RelB-/- mice, indicating that RelB does not play a major role in the regulation of these genes (47,294).

1.2 IxB regulatory proteins

The IkB family of proteins is defined by its ability to interact with NF-kB/Rel subunits and by the presence of between five and seven repeats of a 33 amino acid sequence termed the ankyrin motif (reviewed in 22) (Figure 2). The ankyrin motif mediates sequestration of NF-kB in the cytoplasm, possibly in association with cytoskeletal proteins (234). IkB α (112) is the most extensively studied protein in this family, but other members include IkB β (282), IkB γ (129,171) and the proto-oncogene *bcl-3* (92.113,209). The full length precursors NFkB1/p105 and NFkB2/p100 also have demonstrated inhibitory capabilities, not only over their own amino termini, but also through interaction with c-Rel and RelA (227). There is specificity in the interaction between the inhibitory subunits and the DNA-binding members; IkB α and IkB β exclusively inhibit dimers containing RelA and c-Rel, whereas IkB γ and Bcl-3 preferentially interact with NF-kB1/p50 and/or NF-kB1/p52 -containing dimers (reviewed in 22).

1.3 NF-kB activation via IkB degradation

Rapid activation of NF- κ B involves degradation of I κ B α and/or I κ B β . I κ B α has a half life of 1-2h when complexed with NF- κ B in unstimulated cells (226,260,273). I κ B α is constitutively phosphorylated at multiple sites in the carboxyl terminal PEST domain by casein kinase II (CKII); phosphorylation at these sites is responsible for the short half life of the protein (17,165,184). Activating agents, such as virus infection, cytokines

Figure 2. The IxB family. Members of the IxB family have in common the ability to bind NF-xB proteins and contain multiple copies of the ankyrin repeat domain (black boxes). Despite the presence of the Rel homology domain (cross-hatched pattern), the precursor proteins NFxB1/p105 and NFxB2/p100 do not bind DNA. IxBy comprises the C-terminal end of NFxB1/p105. IxBy is encoded by *nfkb1*, but its mRNA is generated by alternative splicing.



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(TNF- α , IL-1), lipopolysaccharide (LPS) and differentiation inducing agents accelerate the degradation of cytosolic IxB α , thereby promoting release and nuclear translocation of NF- κ B/Rel dimers (Figure 3). Following inducer mediated stimulation, IxB α becomes hyperphosphorylated, detectable in immunoblots as a slowly migrating form, sensitive to phosphatase treatment (23,44). Hyperphosphorylation occurs at the N-terminus of the molecule and is a signal for subsequent ubiquitination and degradation by the 26S proteasome. Neither hyperphosphorylation (5.86,168,284) nor ubiquitination (52) impairs the ability of IxB α to associate with NF- κ B. Moreover, only hyperphosphorylated IxB α is a target for degradation by an *in vitro* reconstituted ubiquitin-proteasome system (6). Phosphorylation and subsequent degradation via the ubiquitin-proteasome degradation pathway are therefore key events in NF- κ B liberation and nuclear translocation (5.6.23,44,52,86,168,284).

An important question with regard to the regulation of NF- κ B transcriptional activity concerns the signaling pathways that stimulate I κ B α phosphorylation and degradation. It remains unclear whether different stimuli trigger multiple independent, signaling pathways or whether signaling events converge upon a common host kinase activity that stimulates phosphorylation and subsequent degradation of I κ B α . A number of potential kinases have been identified which are capable of phosphorylating I κ B α *in vitro*. Double stranded RNA dependent protein kinase (PKR) phosphorylates I κ B α *in vitro* (150) and antisense RNA complementary to PKR blocks NF- κ B activation induced by dsRNA (178). The role of PKR appears to be restricted to dsRNA stimulation, since antisense PKR RNA does not block TNF- α induced NF- κ B activity. TNF- α stimulation appears to involve at least three kinases: Raf-1, the ζ isoform of protein kinase C (PKC) and casein kinase II (CKII) (71,72,85,164,170,297). In vivo, the only kinase identified to date that physically interacts with and phosphorylates I κ B α is CKII. CKII phosphorylates a cluster of sites within the C-terminal PEST domain and mutation of the CKII sites in I κ B α results in a protein with increased intrinsic stability (17,165,184). Figure 3. Regulation of the NF- κ B/I κ B α pathway. Activators of NF- κ B trigger signal transduction cascades that result in hyperphosphorylation of I κ B α (steps 1-2). Hyperphosphorylation is a signal for I κ B α ubiquitination (step 3) and subsequent degradation by the 26S proteasome (step 4). Following I κ B α degradation, NF- κ B translocates to the nucleus and activates NF- κ B dependent cytokine genes. Also activated are components of the NF- κ B pathway such as *Ikba* (I κ B α), *nfkb1* (p105) and *nfkb2* (p100) (step 5). Newly synthesized I κ B α can retain NF- κ B in the cytoplasm or may move to the nucleus and dissociate NF- κ B/DNA complexes, thereby exerting a direct inhibition on gene expression. Newly synthesized p105 and p100 proteins can also retain NF- κ B proteins such as ReIA in the cytoplasm (step 6). p105 and p100 are proteolytically cleaved to generate p50 and p52 respectively (steps 7-8) which can localize to the nucleus or be retained in the cytoplasm, complexed to I κ B α .



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Recent studies have demonstrated that substitution of serine 32 and/or 36 to alanine abolishes inducer mediated phosphorylation and degradation of $I\kappa B\alpha$ (42,43,52,300). These residues thus represent the critical signal response phosphoacceptor sites of multiple activators of NF- κ B, including TNF- α , LPS and Tax protein of HTLV-1. Although both Ser-32 and Ser-36 represent consensus CKII sites, no *in vivo* evidence for CKII phosphorylation within the signal response domain has been obtained. Recently, a new multisubunit kinase was discovered. This kinase is activated by ubiquitination and interestingly can phosphorylate $I\kappa B\alpha$ residues S32 and S36 *in vitro* (53). The resolution of the signaling events involved in $I\kappa B\alpha$ regulation will require further characterization of the kinase activity involved in signal induced phosphorylation of $I\kappa B\alpha$.

Mice deficient in $I\kappa B\alpha$ undergo normal embryonic development but survive only for a few days after birth: the animals present with skin defects, extensive granulopoiesis, severe runting, and small liver, spleen and thymus (25). Nevertheless, the major hematopoietic lineages are formed in absence of $I\kappa B\alpha$. As expected, NF- κB activity is augmented in $I\kappa B\alpha$ -/- mice especially in hematopoietic cells. In thymocytes, expression of G-CSF, macrophage inflammatory protein 2 and VCAM-1 is augmented, whereas expression of GM-CSF, IL-2, IL-2R- α , IL-6 and c-myc is not altered in the $I\kappa B\alpha$ knock-out (25).

IkBβ was recently cloned and characterized (282). Structurally, IkBβ contains six ankyrin repeat regions and a putative C-terminal PEST domain; interestingly, several potential CKII sites are conserved in the IkBβ C-terminal PEST domain. A primary biological difference between IkBα and IkBβ is the response to different inducers of NFkB activity. IkBβ is rapidly degraded upon LPS or IL-1 stimulation, but neither TNF-α nor phorbol ester stimulation induce IkBβ degradation to the same extent. Conversely, rapid IkBα degradation is induced by all four stimuli (25,282). Also, unlike IkBα, IkBβ expression is not induced by NF-kB (25,282). Interestingly, the slower response of IkBβ to TNF-α induced degradation does not prevent NF-kB DNA binding activity to be activated in $I\kappa B\alpha$ knockout fibroblasts (25). These results suggest that unlike $I\kappa B\alpha$, $I\kappa B\beta$ is not utilized for regulating rapid responses but for responding to persistent signals that result in a more permanent activation of gene expression (282).

Proteolytic processing of the precursors NFxB1/p105 and NFxB2/p100 into their active p50 and p52 forms is an additional level at which NF-xB DNA-binding activity may be controlled (Figure 3) (reviewed in 12, 238, 281, 288). For example, p105 and p100 exist complexed to other rel family members such as RelA. c-Rel and proteolytically released p50. Proteolytic cleavage of the carboxyl terminal of p105 activates DNA-binding p50-p50, p50-c-Rel and p50-RelA dimers. This mechanism, however, is not likely to result in a rapid appearance of NF-xB in the nucleus but may contribute to the restoration of the cytoplasmic NF-xB/IxB pool following induction (158) (Figure 3).

NF- κ B mediated transcription is down regulated by *de novo* synthesis of IkB α which interacts with nuclear or cytoplasmic NF- κ B subunits in transit to the nucleus (24). The absence of I κ B α in embryonic fibroblasts allows active NF- κ B DNA binding activity to persist longer following TNF- α stimulation. Increased levels of I κ B α may also dissociate protein DNA complexes in the nucleus and restore the cytoplasmic pool of latent NF- κ B/I κ B complexes (7). Furthermore, transcriptional upregulation of *nfkb1*. *nfkb2*, *c-rel*, *bcl-3* and *Ikba* (I κ B α) genes by NF- κ B provides an autoregulatory mechanism to restore intracellular pools of latent NF- κ B (12,238,281,288).

2. Inhibitors of NF-KB activation.

The central role of the NF- κ B/I κ B pathway in the regulation of genes involved in the inflammatory, immunoregulatory and stress responses has prompted intense interest in NF- κ B molecular biology, as well as in strategies to control NF- κ B activation and the subsequent downstream regulatory processes. Recent information concerning the diverse inhibitors of NF- κ B and cytokine activation are summarized below.

2.1 Transdominant IxBa mutants.

Studies aimed at determining the structural requirements for inducer mediated degradation of IkBa have demonstrated that substitution of serine 32 and/or serine 36 to alanine blocks inducer mediated degradation and phosphorylation by multiple activators (42,43,300). Such mutants are also resistant to ubiquitination and degradation by the proteasome (52,250) but still associate with ReIA (42). Thus, mutations in serine 32 and serine 36 generate IxBa transdominant mutants that function to interfere with NF-xB activation by sequestering NF-xB subunits within a signal independent complex (42,43,272,300). Substitutions of lysine 21 and 22 to arginine residues (K21R/K22R) prevent IkBa ubiquitination in vitro indicating that these residues function as the sites on which ubiquitination occurs (14.250). In vivo, K21R/K22R mutants are phosphorylated but not degraded following stimulation, confirming that hyperphosphorylation precedes ubiquitination in the signal transduction cascade (14,52,250). As opposed to wild type IxB α , K21R/K22R mutants interfere with NF-xB dependent gene activation by TNF- α , phorbol 12-myristate 13-acetate (PMA)/ionomycin (a calcium ionophore) or the human T cell leukemia virus type I (HTLV-I) Tax protein (14,52,250). K21R/K22R IκBα mutants also potentially behave as transdominant inhibitors of the NF- κ B pathway.

2.2 Protease inhibitors

The first protease inhibitors shown to block NF- κ B activation by blocking I κ B α hyperphosphorylation and degradation were alkylating agents including phenylalaninechloromethyl ketone (PCK), N-tosyl phenylalanine chloromethyl ketone (TPCK) 3,4dichloroisocoumarin (DCIC), and N α -p-tosyl-L-lysine chloromethyl ketone (TLCK) (54,86,116,200). However, these inhibitors are not particularly specific since they also react with NF- κ B proteins and abolish DNA-binding activity (86,200). Moreover, PCK and TPCK also block the binding activity of other transcription factors including AP-1, CREB and Oct-1 (200). The serine protease inhibitors 4-(-2-aminoethyl)- benzenesulfonyl fluoride (AEBSF) and N-benzoyl-L-tyrosine ethyl ester (BTEE) block NF- κ B activation by interfering with degradation and hyperphosphorylation of I κ B α . Importantly, these inhibitors do not abolish NF- κ B binding activity by chemically modifying NF- κ B proteins. AEBSF and BTEE may interfere with a step upstream of I κ B α degradation in the signal transduction cascade since they block I κ B α phosphorylation (86).

2.2.1 Peptide aldehyde inhibitors.

Two major classes of proteases are known to operate outside of the lysosome where IsBa is degraded. These are the calpains (Ca++-dependent cysteine proteases) and the multicatalytic proteinase complex (proteasome). Conjugation to multiple molecules of ubiquitin targets protein to degradation by the 26S proteasome complex (56). The catalytic activities of the core 20S proteasome are diverse and include chymotrypsinlike, peptidylglutamyl peptide-hydrolyzing, trypsin-like and caseinolytic activities. Peptidyl aldehydes are both serine and cysteine protease inhibitors that function as competitive inhibitors of various proteases through binding to their active sites. Peptidvl aldehydes are perhaps the most specific calpain and proteasome inhibitors presently available. The first peptidyl aldehyde inhibitors shown to inhibit IxB α degradation and NF-kB activation were calpain inhibitors; N-acetyl-LL-norleucinal (calpain inhibitor I) but not N-acetyl-LL-methioninal (calpain inhibitor II), inhibits IkBa degradation induced by TNF- α (43,73,192), IL-1 (73), LPS, and PMA/ionomycin (43,300). Calpain inhibitor I is a better inhibitor of the chymotrypsin-like activity of the proteasome than calpain inhibitor II (84), indicating a correlation between inhibition of the chymotrypsinlike activity of the proteasome and inhibition of $I\kappa B\alpha$ degradation. Two other potent inhibitors of the chymotrypsin-like activity, N-benzyloxycarbonyl-LL-phenylalaninal (Z-LLF) (73) and N-benzyloxycarbonyl-IE-(Ot-Bu)A-leucinal (PSI) (284,302) also inhibit TNF- α induced IxB α degradation.

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Two peptidyl aldehydes have been developed that are more efficient in inhibiting chymotrypsin-like activity of the proteasome than calpain inhibitor I and therefore are more specific (230). These inhibitors, N-benzyloxycarbonyl-LL-norvalinal (MG115) and N-benzyloxycarbonyl-LL-leucinal (MG132), efficiently block IxB α degradation induced by TNF- α (213), calyculin A (271) or the HTLV-1 Tax protein (177). Peptidyl aldehydes specifically block IxB α degradation but not IxB α hyperphosphorylation and do not cause chemical inactivation of NF-xB proteins (73,177,192,271,284,300). These inhibitors thus represent specific tools with which to dissect the signal transduction pathway leading to NF-xB activation. These agents also have potential as anti-inflammatory or immunomodulatory drugs since they efficiently block expression of NF-xB regulated genes. Peptidyl aldehydes also block adherence of monocytes to endothelial cells activated by TNF- α by blocking E-selectin, VCAM-1 and ICAM-1 expression (224).

2.3 Immunosuppressive agents

2.3.1 Glucocorticoids.

Glucocorticoids are secreted by the adrenal cortex and play a critical role in cellular metabolism by stimulating gluconeogenesis, promoting amino acid mobilization from muscles and mobilization of fatty acid from adipose tissue. The additional immunosuppressive activities of glucocorticoids have resulted in the extensive clinical use of steroids as immunosuppressive and anti-inflammatory agents (37). Glucocorticoids, like other steroid hormones, enter the cell by simple diffusion and bind to the glucocorticoid receptor (GR) which is localized in the cytoplasm in complex with heat shock protein Hsp90 and immunophilin p56. Upon ligand binding, activated GR dissociates from Hsp90 which presumably masks the nuclear localization sequence of GR, and translocates to the nucleus (128,308). Activated GR binds to a specific DNA recognition sequence, the glucocorticoid response element (GRE) and stimulates
transcription of steroid hormone responsive genes. Activated GR may also repress gene transcription, either by binding to a DNA sequence termed the negative GRE (nGRE) as in the case of the prolactin gene, or by interfering with essential transcription factors such as AP-1 (20).

Recent experiments have also demonstrated that glucocorticoids interfere with the transcription factor NF- κ B. A number of cytokine genes regulated by NF- κ B are repressed by glucocorticoids including genes encoding GM-CSF, IL-2, IL-3, IL-2R- α , IL-6, IL-8 and TNF- α (4,60,66,140,198,199,222,286). Inducible nitric oxide synthase (iNOS) production in vascular endothelial cells is also inhibited by glucocorticoids; adhesion molecules, E-selectin and ICAM-1 are also inhibited by glucocorticoids, leading to reduced adhesion of endothelial cells to leukocytes (189, 203 and references therein). Glucocorticoids are capable of inhibiting NF- κ B DNA binding activity induced by numerous agents such as TNF- α , IL-1, 12-O-tetradecanoyphorbol-13-acetate (TPA), anti-CD3 antibodies PMA/phytohemagglutinin (PHA), ionomycin/ TPA and LPS (8,248).

The inhibition of NF- κ B activity by the synthetic glucocorticoid dexamethasone (Dex) requires the presence of the glucocorticoid receptor (48) and is dependent on both transcription (8) and protein synthesis (8,248). Recent studies demonstrate that Dex activates the transcription and synthesis of I κ B α (8,248); Dex-GR appears to induce *Ikba* transcription directly, since cycloheximide does not inhibit this process (248) (Figure 4). However, an *Ikba* promoter construct extending to position -600 bp is not activated by Dex indicating that a putative GRE must be located further upstream or within the gene (248). Dex also induces *nfkb1* gene transcription and NF κ B1/p50 homodimer DNA binding; overexpression of NF κ B1/p105 can repress NF- κ B by sequestering it in the cytoplasm. Moreover, NF κ B1/p50 homodimer are poor transactivatiors and may repress transcription by competing with the binding of active p50-ReIA heterodimers (19).

Figure 4. Inhibition of NF- κ B activation by glucocorticoids. A. In unstimulated cells, both the glucocorticoid receptor (GR) and NF- κ B are retained in the cytoplasm by heat shock protein 90 (Hsp90) and I κ B α , respectively. Upon TNF- α stimulation, I κ B α is degraded, NF- κ B then translocates to the nucleus and transactivates *Ikba* (I κ B α) gene expression. B. Upon dexamethasone treatment (Dex), GR binds Dex and dissociates from Hsp90. Dex-activated GR can transactivate *Ikba* gene expression through a putative glucocorticoid responsive element (GRE). C. Upon Dex and TNF- α treatment, Dex-mediated *Ikba* transcription results in a cytoplasmic distribution of NF- κ B due to I κ B α overexpression. Also Dex-GR can interact with ReIA, thereby preventing nuclear NF- κ B-mediated gene activation. Thick arrows refer to predominantly active steps in the pathway. Thin arrows refer to less active steps in the pathway.



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Induction of $I \times B \alpha$ and $N F \times B 1/p 105$ expression may partly explain the inhibition of NF- κ B activity by glucocorticoids; however other studies demonstrate that GR interacts directly with RelA and interferes with RelA transactivation (Figure 4). RelA can be co-precipitated with GR in vivo (48,223,249) and overexpression of RelA prevents GR transactivation of a reporter gene under the control of GRE (48,223,249). Based on these studies it appears that the interaction between GR and ReIA is sufficiently strong to interfere with the binding and transactivation by these two transcription factors. Moreover, in cells transfected with both RelA and GR, Dex treatment results in a significant loss of nuclear RelA (249), suggesting that activated GR can sequester RelA in the cytoplasm. However, Dex causes a dramatic translocation of RelA from the nuclei of TNF- α stimulated cells to the cytoplasm, while GR remains nuclear (8), suggesting that Dex may exert its inhibitory activity in the nucleus by dissociating NF-xB DNA complexes (249). Interestingly, GR can bind RelA even in the absence of Dex (249) whereas in transfection experiments, repression depends on the presence of both Dex and GR. The requirement for Dex may be to localize GR in the nucleus where it can interfere with RelA.

The steroid analog RU486 permits GR dissociation from Hsp90 and translocation to the nucleus, but blocks the transactivation potential of GR (249). Although less effective than Dex, RU486 can still inhibit NF- κ B activity induced by TNF- α (48) or RelA overexpression (249). Moreover, Dex can inhibit NF- κ B activity induced by TNF- α through a mutant GR which cannot bind GRE (48). If I κ B α is induced by Dex through a GRE, RU486 and the mutant GR are not expected to activate I κ B α expression, thus supporting the idea that I κ B α induction is not the only mechanism through which Dex inhibits NF- κ B.

2.3.2 Aspirin.

Aspirin and salicylate are nonsteroidal anti-inflammatory drugs. Aspirin is probably the oldest synthetic drug in the pharmacopeias today. However, little is known about its mode of action other than its inhibitory activity for cyclo-oxygenase enzymes (99). Recently, aspirin and salicylate were shown to inhibit NF- κ B activity and I κ B α degradation in doses prescribed for rheumatoid arthritis (148). However, the inhibition by aspirin does not appear to be specific for NF- κ B. Under similar conditions, salicylate is also capable of inhibiting CREB (cAMP responsive element binding proteins) and AP-1 transcription factors. Also, salicylate exerts a profound inhibition on cellular protein kinase activity and thus may inhibit the kinase involved in I κ B α phosphorylation (91). Other general protein kinase inhibitors such as staurosporin also inhibit NF- κ B activation by PMA, TNF- α and IL-1 (133).

2.3.2 Prostaglandin E2.

Prostaglandin E2 (PGE2) is a lipid mediator derived from arachidonic acid metabolism when arachidonic acid is released from cellular phospholipids. PGE2 is produced during a cell mediated immune response and exerts a negative feedback on immune function via inhibition of cytokine release. PGE2 can stimulate IL-4 production which negatively regulates the expression of a number of cytokines (285). PGE2 binds to a surface receptor and stimulates cAMP production which then activates the protein kinase A (PKA) pathway. Elevated intracellular cAMP levels inhibit IL-2 production and suppress proliferation of T lymphocytes (138). Following TPA/ionomycin stimulation of T-cells, forskolin, which stimulates intracellular cAMP levels, mimics PGE2 by increasing transcription from the IL-4 promoter (206) and decreasing transcription from the IL-2 promoter (51,206). Repression of the IL-2 promoter by PGE2 or elevated intracellular cAMP levels is mediated through the NF- κ B site in the IL-2 promoter (51,206). Furthermore, PGE2 inhibits activation of NF- κ B binding activity induced by TPA/ionomycin (51,206) or anti-CD3 antibody (292) in T cells. PGE2 appears to inhibit RelA nuclear translocation but not c-Rel translocation, perhaps due to a decreased rate of $I\kappa B\alpha$ degradation (206).

2.3.3 FK506 and CsA.

FK506 and cyclosporin A (CsA) are widely used immunosuppressive drugs capable of suppressing T-cell dependent antibody production, graft versus host reactivity and delayed type hypersensitivity in vivo. These drugs suppress T-cell proliferation, IL-2 production and other growth promoting cytokines in vitro (194). CsA and FK506 inhibit antigenic stimulation of T cells by inhibiting the activation of the T cell specific transcription factor NFAT which is essential for IL-2 expression (79,221). A number of NF- κ B regulated genes that are not known to be regulated by NFAT are also sensitive to CsA or FK506 repression including genes encoding IL-2R- α , NFxB1, IL-8, E-selectin and iNOS (57,154,182,210,219). CsA and FK506 inhibit antigenic stimulation of NF- κ B DNA binding activity in T cells (182,210). Antigenic stimulation can be mimicked by co-treatment with phorbol esters and calcium ionophores which provide a protein kinase C dependent signal and calcium dependent signal, respectively. CsA and FK506 inhibit NF-kB activity stimulated with PMA/ionomycin or other stimuli which resemble PMA/ionomycin costimulation (157,293). However, CsA and FK506 do not inhibit NF-kB induction by PMA alone, and ionomycin alone does not induce NF-kB (90,157,181,270). Therefore it appears that intracellular calcium release participates in the activation of NF-kB following antigenic stimulation and is the target of CsA and FK506 inhibition.

The role of intracellular calcium release in NF- κ B activation involves the calcium and calmodulin-dependent protein phosphatase, calcineurin which is the regulatory target of CsA and FK506 inhibition. Overexpression of calcineurin or a calcium independent mutant of calcineurin, synergizes with PMA or c-Rel

overexpression to activate NF- κ B dependent transcription via $I\kappa B\alpha$ degradation (90,267.287) (Figure 5). CsA and FK506 bind the immunophilins - cyclophilin and FK506 binding protein, respectively - and together form a complex that can bind and inhibit the phosphatase activity of calcineurin (194). Inhibition of calcineurin may thus block the dephosphorylation of NFAT which is necessary for its nuclear translocation (242), as well as an independent cascade of events that potentiates $I\kappa B\alpha$ degradation (Figure 5).

One may speculate that calcium release contributes to the induction of NFATdependent cytokine gene expression and that cytokine release thereby stimulates NF- κ B in an autocrine fashion. Recent observations however indicate that calcium release directly participates in NF- κ B activation since the calcium dependent activation of NF- κ B is protein synthesis independent and CsA inhibits NF- κ B induction within minutes of anti-CD3 antibody addition. Moreover, NF- κ B binding activity is not inhibited by anti-TNF- α antibodies (219) or anti-IL-2R- α antibodies (182), demonstrating that calciumdependent activation of NF- κ B does not occur indirectly through synthesis of IL-2 or TNF- α . However, sustained NF- κ B induction observed up to 24h after anti-CD3 antibody challenge depends on protein synthesis and TNF- α autocrine stimulation since it can be blocked by cycloheximide and anti-TNF- α antibodies (219).

2.4 Antioxidants

Oxidative stress refers to any biological process which results in an increase in intracellular reactive oxygen intermediates (ROI). ROI include super anion O_2 , hydrogen peroxide H₂O₂, and hydroxyl radical (·OH). ROI normally arise as a consequence of aerobic respiration in mitochondria when incomplete reduction of the electron acceptor dioxygen (O₂) occurs. Elevated ROI occur following a number of cellular injuries including bacterial or viral infection, inflammation, heat shock, ionizing and UV irradiation as well as exposure to a wide variety of chemicals (for review see

Figure 5. Inhibition of NF- κ B activation by FK506 and cyclosporin. A. A. Antigen stimulation of T cells can be mimicked by ionomycin and PMA stimulation. Ionomycin increases intracellular calcium release which stimulates the calmodulin (CM)calcium-dependent protein phosphatase, calcineurin. PMA stimulation induces $I\kappa B\alpha$ degradation through a signal transduction cascade involving protein kinase C, reactive oxygen intermediates and protein kinases or phosphatases. PMA stimulation synergizes with activated calcineurin in the degradation of $I\kappa B\alpha$, resulting in the appearance of active nuclear NF- κB . B. FK506 and cyclosporin A bind immunophilins (IP) which subsequently bind to calcineurin. The resulting complex is inactive, thus compromising the calcium dependent pathway participating in the degradation of $I\kappa B\alpha$ degradation. Thin arrows refer to the reduced NF- κB activation occuring upon ionomycin and PMA stimulation in the presence of cyclosporin A or FK506, as opposed to strong NF- κB activation occuring upon ionomycin and PMA stimulation in the absence of these drugs (thick arrow).



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259). ROI elevation can be specifically induced by treating cells with hydrogen peroxide (H₂O₂). After membrane diffusion, H₂O₂ in the presence of superoxide anions (O₂⁻) and a metal catalyst, is converted by the non enzymatic Haber Weiss reaction into hydroxyl radicals (·OH): [O₂⁻ + H₂O₂ -> O₂+ ·OH + OH⁻] (289). Interestingly, H₂O₂ activates NF-κB DNA binding activity and NF-κB regulated genes such as IL-2, IL-2R-α and VCAM-1 in several cell types (245,257-259,265,276). Since intracellular H₂O₂ and O₂⁻ have been observed upon treatment with known NF-κB activators such as IL-1, TNF-α (186), and LPS (257), it appears that ROI are part of a physiological signal transduction pathway leading to NF-κB activation.

Reduced glutathione (GSH: L- γ -glutamyl-L-cysteinyl glycine) protects the cell from oxidative stress by converting H₂O₂ into H₂O: [GSH + H₂O₂ -> GSSH + 2H₂O], catalyzed by glutathione peroxidase: the reverse reaction is catalyzed by NADPHdependent glutathione reductase. A decrease in the ratio of cellular GSH/GSSH is an indicator of an oxidative stress (259). Accordingly, stimulation of glutathione peroxidase inhibits NF- κ B activation by H₂O₂, TNF- α and PMA (246). Also, L-cysteine - the precursor of GSH - inhibits transcriptional activation of the HIV LTR by NF- κ B inducers such as TNF- α , IL-6 or PMA (136,190). Another related antioxidant, N-acetyl cysteine (NAC) which increases GSH levels in the cell also inhibits induction of NF- κ B by H₂O₂, cycloheximide, dsRNA, IL-1, TNF- α , PMA and LPS (259).

Pyrrolidine dithiocarbamate (PDTC) has been shown to inhibit NF- κ B at concentrations more than two logs lower than NAC (258). PDTC is both a metal chelator and an antioxidant and absorbs metal ions required for the Haber Weiss reaction, thus protecting the cell from hydroxyl radicals (·OH). The iron chelators desferal or O-phenanthroline also block induction of NF- κ B binding activity induced by H₂O₂ (257). The dual activities of PDTC may account for its higher efficiency. Expression of NF- κ B regulated genes encoding IL-2, IL-2R- α , iNOS, c-myc, VCAM-1 and ICAM-1 is blocked by PDTC treatment. Other antioxidants have been successfully

used to inhibit NF- κ B activity or expression of NF- κ B regulated genes including butylated hydroxyanisole (BHA), tetrahydropapaveroline (THP), nordihydroguaiaretic acid (NDG), 10,11,dihydroxyaporphine (DHA), alpha-lipoic acid, 2,3-dihydroxybenzoic acid (DBH) and topoxalin (2,81,132).

The cell has also developed mechanisms for the elimination of superoxide anions O_2 . Superoxide anions are converted into hydrogen peroxide by superoxide dismutases (SOD): $[2 O_2^{-} + 2H^+ -> H_2O_2 + O_2]$ and potentially damaging hydrogen peroxide is then neutralized immediately by peroxidases or catalases. The mitochondrial matrix enzyme manganese-dependent-SOD (Mn-SOD) is inducible by IL-1 and TNF- α (108). Mn-SOD is a NF- κ B regulated gene (67) that is likely to exert a negative feedback on NF- κ B by controlling ROI in the cell. Overexpression of HIV-1 Tat which potentiates TNF- α activation of NF- κ B activity, inhibits Mn-SOD expression. This inhibition correlates with a decrease in the GSH/GSSH ratio, suggesting that Mn-SOD acts as an antioxidant with respect to NF- κ B activation (295).

2.4.1 Antioxidants block IxBα phosphorylation.

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The antioxidant PDTC blocks stimuli-induced I κ B α degradation by preventing I κ B α phosphorylation (23,116,273,284) suggesting that ROI act upstream of I κ B α phosphorylation in the signal transduction cascade. However, it remains to be determined whether ROI elevation leads to activation of an I κ B α kinase or to the inhibition of a protein phosphatase that allows constitutive kinases to phosphorylate I κ B α . In support of the latter hypothesis, okadaic acid and calyculin A activate NF- κ B at low concentration (25-50 nM) by inhibiting protein phosphatase 1 and 2A (271,275). Neither NAC (275) nor PDTC inhibits NF- κ B activation under these conditions (271) indicating that ROI act upstream of a putative protein phosphatase inhibition step. Contrasting with these observations, NF- κ B activity induced by high concentration of OA or calyculin A is inhibited by L-cysteine (188) or PDTC (253). Importantly, OA and calyculin A when used in μ M concentrations induce H₂O₂ production (253); at high concentration, okadaic acid may activate NF- κ B by generating an oxidative stress which can be inhibited by antioxidants.

2.4.2 Nitric oxide.

Nitric oxide is an important endogenous regulator acting in a variety of biological processes including tumor suppression, vasodilatation and neurotransmission (252). NO is naturally generated from the conversion of L-arginine into L-citrulline and can be artificially induced by the addition of a NO-generating compound (NGC) to cells. IL-1 induced monocyte adherence to vascular endothelial cells is inhibited by the NGC S-nitrosolo-glutathione (GSNO). GSNO exerts this effect by repressing a number of NF- κ B regulated genes. Synthesis of the cytokines IL-6 , IL-8 and M-CSF, the monocyte chemoattractant protein-1 (MCP-1), and expression of the adhesion molecules E-selectin and VCAM-1 is repressed by GSNO in IL-1 or TNF- α stimulated vascular endothelial cells (69,215,311). GSNO inhibits NF- κ B DNA binding and transactivation through the NF- κ B element of the VCAM-1 and M-CSF promoter (69,214,311). Inducible nitric oxide synthase is a NF- κ B regulated gene (97) which suggests that it may exert a negative feedback on NF- κ B by increasing NO production. Accordingly, inhibition of endogenous NO production by L-N-monomethyl-arginine (L-NMA) treatment stimulates NF- κ B activity in monocytes and vascular endothelial cells (215).

In vascular endothelial cells, GSNO activates $I\kappa B\alpha$ mRNA synthesis and an $I\kappa B\alpha$ promoter construct extending to position -1.6 kb is responsive to NO. As a result of $I\kappa B\alpha$ overexpression, more $I\kappa B\alpha$ may be bound to NF $\kappa B1/p50$ -RelA heterodimer in cells stimulated by TNF- α and treated with GSNO than in TNF- α stimulated cells (214). Since other antioxidants have not been shown to induce $I\kappa B\alpha$ expression, NO may represent a distinct class of NF- κB inhibitors. Immunosuppressive drugs, antioxidants and steroid hormones act as potent inhibitors of NF- κ B activation by targeting specific steps in the NF- κ B/I κ B activation cascade (Figure 6). These compounds have provided insight into the complex molecular control of immunoregulatory gene expression. In addition, they promise to play an increasingly important role in suppressing pathological inflammatory responses and may provide a pharmacological basis for interfering with many diseases including cancer, AIDS and infectious diseases (12).

3. The role of NF-xB in cell growth regulation and oncogenesis

Several lines of evidence link the NF- κ B family of transcription factors to cell growth regulation and leukemogenesis: (i) *bcl-3* and *nfkb2* are involved in chromosomal translocations in a subset of human B-cell lymphomas and other malignancies (205,209): (ii) *v-rel*, the avian homologue of *c-rel*, is responsible for the tumorigenicity of the avian reticuloendotheliosis virus REV-T (36); (iii) the Epstein-Barr virus and the human T cell leukemia virus type 1 depend on NF- κ B to confer selective growth advantage to infected host cells (121,191); and (iv) tumor necrosis factor alpha, a strong activator of NF- κ B, stimulates proliferation of certain cells (49).

3.1 Chromosomal translocations

The discovery of the *nfkb2* gene resulted from the characterization of the t(10:14)(q24:q32) chromosomal translocation found in a B-cell Non-Hodgkins lymphoma. *nfkb2* was originally designated as *lyt-10* for lymphocyte translocation on chromosome 10. The chromosomal translocation juxtaposes the immunoglobulin C α_1 locus to *nfkb2* and results in the substitution of the 3' end of *nfkb2* with immunoglobulin sequences (205,251). The encoded fusion protein lyt-10-C α_1 retains the RHD of NF κ B2 but lacks the ankyrin repeats. Unlike NF κ B2/p100, the fusion protein lyt-10-C α_1 , binds NF κ B DNA *in vitro* (205), suggesting that removal of the inhibitory ankyrin repeats

Figure 6. Inhibitors of NF- κ B activation target the I κ B α molecule. Antioxidants, aspirin, cyclosporin A and FK506 interfere with events in the signal transduction cascade leading to I κ B α hyperphosphorylation (86,148,259). Substitution of serine 32 and 36 to alanine blocks I κ B α hyperphosphorylation (42,43,272,300) while substitution of lysine 21 and 22 to arginine blocks I κ B α ubiquitination (14,250). Peptidyl aldehyde proteasome inhibitors specifically interfere with the degradation of I κ B α by the proteasome (73,177,192,271,284,300). Glucocorticoids interfere with NF- κ B binding activity by binding to RelA (48,223,249). Nitric oxide and glucocorticoids interfere with NF- κ B proteins (8,214,248).



generates a constitutively active form of NF κ B2. Structural alterations of *nfkb2* have since then been identified in B cell chronic lymphocytic leukemia (B-CLL), cutaneous B cell lymphoma, multiple myeloma, and cutaneous T cell lymphomas (104,208,280). These alterations frequently affect the carboxy terminal ankyrin repeats. Interestingly, a T lymphoma cell line carrying a truncated *nfkb2* gene was shown to have elevated NF- κ B binding activity (208), suggesting that loss of the NF κ B2/p100 I κ B activity may contribute to elevated NF- κ B binding and the oncogenicity of certain B and T cell lymphomas.

The Bcl-3 protein contains 7 ankyrin repeats and acts as an IkB molecule by inhibiting the DNA binding activity of NFkB1/p50 homodimers (92,142,207,305). Bcl-3 also associates with DNA complexes containing NFkB2/p52 homodimers and provides a strong transactivation function (39). bcl-3 is involved in a chromosomal translocation found in a subset of human B-cell chronic lymphocytic leukemia (CLL) (209). The translocation, t(14:19), juxtaposes the α constant region exons of the immunoglobulin heavy chain locus (IgH gene, on chromosome 14) to the bcl-3 gene on chromosome 19 in a head-to-head fashion without affecting the transcriptional integrity of bcl-3 (185). The translocation however, affects the expression of *bcl-3*: a three to four fold increase in *bcl-3* mRNA levels is found in leukemic cells from CLL patients carrying the translocation compared to leukemic cells from other CLL patients (209). Consequently, bcl-3 overexpression may contribute to tumor development. Interestingly, both translocations involving *nfkb2* and *bcl-3* may result in an increase of NF κ B2-mediated. transcriptional activation. Altered NF-xB gene regulation may confer a selective growth advantage to cells that permits the subsequent acquisition of additional oncogenic mutations.

nfkb1 and *c-rel* have been mapped to the chromosome region 4q24 and 2p14-15 respectively, both regions are rearranged in some lymphoproliferative diseases(176,180). In a cell line derived from a diffuse large cell lymphoma, the 5' end of *c-rel* is fused to the coding region of an unrelated gene. The resulting chimeric transcript encodes a c-Rel related protein containing the RHD but lacking the cytoplasmic anchor and the transactivation domain (176). Interestingly, this protein has a structure similar to the v-Rel oncoprotein.

3.2 The *v-rel* oncogene

v-rel is the transforming gene of the avian reticuloendotheliosis virus (Rev-T) (36) which causes a rapid fatal hematopoietic disease in young birds (163,229). Rev T is replication defective and co-replicates with a helper virus, the reticuloendotheliosis associated virus (Rev-A) (123). Hematopoietic cells from both the spleen and bone marrow are targeted for Rev T transformation (18,123).

v-rel originated from a non-homologous recombination between Rev-A and the *c-rel* proto-oncogene causing the insertion of *c-rel* sequences into the viral *env* gene, as such, *v-rel* was the first member of the NF- κ B/Rel family of transcription factors to be associated with oncogenesis. Subsequently, mutations contributing to the tumorigenic phenotype were positively selected during the replication of Rev-T (268,301). The v-Rel protein contains 11 and 18 aa derived from the *env* sequences which replace 2 aa at the N-terminus and 118 aa at the C-terminus of c-Rel respectively. The substitution at the C-terminus of c-Rel is out-of-frame and results in the removal of the c-Rel cytoplasmic anchor and transactivation domain II (103,228) (Figure 7). v-Rel however retains transactivation domain I which possesses significant transactivation potential (247,283,306). Comparison of v-Rel and c-Rel protein sequences reveals the existence of 13 single aa substitutions and three small internal deletions which are distributed at the N- and C-terminus of the RHD as well as in the transactivation domain I (50,301) (Figure 7).

Upon Rev-T infection, v-Rel becomes the major NF- κ B binding protein in the nucleus. Shortly after infection, I κ B α , NF κ B1 and NF κ B2 expressions are induced and



Figure 7. Structure of c-Rel and positions of mutations in v-Rel. The RHD is divided into a DNA-binding region and a dimerization region. The small black box in the DNA-binding domain represents the sequence which directly contacts DNA. The open elipse indicates the position of the nuclear localization sequence. In the C-terminal region of c-rel, two transactivation domains (horizontal line pattern) overlap with a cytoplasmic anchoring region (vertical line pattern). Regions in which v-Rel differs from avian c-Rel are indicated as follow: black boxes indicates additions of aa encoded by *env*-derived sequences; white elipses indicate the 13 aa substitutions and the three small internal deletions. The mutations are grouped into three clusters by long black ovals. This figure is reproduced from reference (125).

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v-Rel accumulates in the cytoplasm. The predominant cytoplasmic distribution of v-Rel correlates with increased $I\kappa B\alpha$ stability probably through $I\kappa B\alpha$ -v-Rel protein-protein interactions (127). Nevertheless, up to 25% of v-Rel is still found in the nucleus (68).

The predominant cytoplasmic distribution has suggested that v-Rel alters gene expression by an indirect mechanism in which IkB proteins preferentially bind to v-Rel and thereby allow NF- κ B proteins to translocate to the nucleus. Recent reports however, suggest that v-Rel acts by directly binding to enhancers elements. This idea is supported by the following observations: (i) c-Rel is capable of transforming avian cells but with much lower efficiency than v-Rel and deletion of c-Rel transactivation domain II. including the cytoplasmic anchor, is associated with an increased presence of c-Rel in the nucleus and a higher transformation efficiency (125); (ii) a chimeric protein with the central domain of c-Rel replaced with the corresponding v-Rel sequences has an increased oncogenic potential and can bind DNA in vitro even in the presence of $I\kappa B\alpha$ (75): conversely (iii), substitution of the central domain of v-Rel with the corresponding c-Rel sequences not only increases the sensitivity of v-Rel DNA complexes to IxBa but abolishes the oncogenic potential of v-Rel (75); furthermore (iv), a mutation which affects the ability of v-Rel to bind IxBa was shown to localize v-Rel to the nucleus without compromising its ability to transform cells (298). All together, these studies suggest that v-Rel does not need to be localized to the cytoplasm to induce transformation.

Analysis of chimeric proteins between c-Rel and v-Rel demonstrated that mutations at both ends of the RHD and the mutated transactivation domain I, all contribute to the transformation efficiency of v-Rel (75,96,103,125,197,204,247,277). The DNA binding activity mediated by the RHD is essential for transformation (96,103,152,195) and mutated v-Rel proteins that are incapable of dimerizing also fail to transform lymphoid cells. However, many mutations which affect DNA binding will also affect the ability of v-Rel to dimerize (195-197,299). Nonetheless, a mutant that cannot homodimerize

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was shown to transform cells when co-expressed with NF κ B2/p52 (298). This mutant dimerizes with NF κ B2/p52 and the resulting heterodimer binds DNA, indicating that v-Rel must associate with itself or with other NF- κ B/Rel family members to mediate transformation (137).

Supporting the idea that v-Rel alters gene expression directly, unique NF- κ B/DNA complexes are found in cells infected with retroviruses expressing v-Rel but not c-Rel. Mutations at the C-terminal end of the RHD are responsible for the appearance of these unique NF- κ B/DNA complexes. Also, v-Rel has an increased affinity for the MHC class I NF- κ B site when compared to c-Rel suggesting that it may regulate gene expression differently. The mutations at both end of the RHD are responsible for this difference in DNA affinity (124).

The ability of v-Rel to inhibit transcription activation by other Rel proteins in transient gene reporter assays raised the possibility that v-Rel promotes transformation by acting as a *trans*dominant negative mutant of NF- κ B activity (16,130,183,228). However, in some cell types, v-Rel does activate transcription in transient gene reporter assays (59,290). Moreover, expression of v-Rel in cells from avian hematopoietic origins induces expression of I κ B α (127,149), MHC class I (34,126,204) and class II (126,204), HMG 14b (34), and IL-2 R α (126) In particular, v-Rel activates MHC Class I and II, IL-2R α , I κ B α , NF κ B1 and NF κ B2 expression more efficiently than c-Rel (126,127). The fact that v-Rel activates gene expression through protein sequences which overlap those required for cell transformation (131,204,247) suggests that v-Rel may also transform cells by activating the transcription of specific cellular genes.

3.3 Oncoviruses that target the NF-kB pathway

The human T cell leukemia virus type I (HTLV-I) and Epstein-Barr virus (EBV) are example of many viruses that target the NF-kB pathway. Interestingly, recent studies

suggest that HTLV-1 and EBV depend on NF- κ B activation to initiate the property of cellular immortalization.

HTLV-1 is the etiologic agent of adult T cell leukemia (ATL) (80). The Tax protein encoded by HTLV-1 is an activator of NF-κB and is involved in leukemogenesis (for review see 121). Transgenic mice expressing the Tax protein develop fibroblastic tumors. These tumors regress upon treatment with antisense RelA oligodeoxynuclectides, which also inhibit NF-κB activity (147). The correlation between oncogenesis and NF-κB activation is also observed *in vitro*. Rat fibroblasts are transformed by overexpression of wild type Tax but not by mutants lacking the ability to activate NF-κB. Furthermore, co-expression of NFκB2 with Tax inhibits NF-κB activation and blocks transformation of fibroblasts (307). Tax also immortalizes primary T cells *in vitro* and is believed to mediate the initial steps in ATL leukemogenesis (for review see 121). Tax induces T cell proliferation at least in part by up-regulating IL-2 and IL-2Rα expression through NF-κB activation. Accordingly, T cells transformed by Tax are dependent on IL-2 stimulation for growth.

EBV is the major cause of infectious mononucleosis and is associated with a number of lymphoproliferative diseases in immunocompromised individuals (135). EBV immortalizes B-cells *in vitro* and requires expression of the EBV-latent membrane protein (LMP-1) for this property (141). LMP-1 induces *bcl-2* expression which protects B-cells from apoptosis (115). Moreover, LMP-1 also induces NF- κ B activity (109) through I κ B α degradation (118), as well as the expression of several NF- κ B regulated genes including those encoding the adhesion molecules LFA-1, LFA-3 and ICAM-1 (291). Interestingly, unlike wild type LMP-1, LMP-1 mutants that are defective for NF- κ B activation do not transform fibroblasts *in vitro* (191).

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3.4 Induction of cellular proliferation by TNF-a

Tumor necrosis factor alpha is a strong activator of NF- κ B with a wide range of biological activities. In particular, TNF- α induces apoptosis in certain tumor cells but stimulates proliferation of primary fibroblasts and other cell types (49). Recent studies suggest that TNF- α depends on NF- κ B for cell proliferation induction: (i) primary fibroblasts proliferate in response to IL-1 which activates NF- κ B (1); (ii) in senescent fibroblasts, NF- κ B dependent gene expression is poorly induced by TNF- α and proliferation is not stimulated (1); and (iii) two T cell lymphoma cell lines (HuT-78 and K-4) have elevated endogenous NF- κ B activity and secrete high amounts of TNF- α . In these cells, both proliferation and NF- κ B activity is dependent on TNF- α , since anti-TNF- α antibody inhibits both (208).

Recent reports suggest that TNF- α plays a role in hepatocyte proliferation during liver regeneration. Following partial hepatectomy, quiescent hepatocytes undergo one or two round(s) of replication in response to the secretion of a number of cytokines including transforming growth factor- α (TGF- α), hepatocyte growth factor (HGF) and epidermal growth factor (EGF) (83). One of the earliest events of liver regeneration is the activation of NF- κ B, which may occur in response to local secretion of TNF- α (63,87,279). NF- κ B activity appears to play a role in hepatocyte proliferation by increasing responsiveness to growth factor(s). Indeed, minimal hepatectomy (MH) induces NF- κ B activity but not hepatocyte proliferation. However, MH renders hepatocytes responsive to TGF- α and HGF (174). Strikingly, normal hepatocytes proliferate in response to EGF and insulin-selenium-transferrin if they are first treated with TNF- α (74). How NF- κ B primes hepatocytes to respond to growth factors is yet undefined but the proto-oncogenes c-myc and GRO α are thought to play a role since their expression is regulated by NF- κ B and is activated during liver regeneration (162,193,303). Interestingly, mice deficient in RelA die during embryonic development

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with massive apoptotic liver cell death (26), suggesting that NF- κ B plays an important role in the growth regulation of hepatocytes.

Addition of serum to the media of quiescent immortalized fibroblasts induces cellular proliferation and NF- κ B activity (15). Moreover, NF- κ B genes *nfkb1*(40), *nfkb2* (38), *c-rel* (45), *relb* (243) and *lkba* (112,142,209,279) are immediate-early response genes. However, TNF- α induces NF- κ B activity in cells at different stages in the cell cycle but does not affect the cell cycle profile of G0 arrested immortalized fibroblasts (78). Therefore, NF- κ B activity may not be a sufficient signal for cell entry in the cell cycle.

All together these studies clearly demonstrate that the NF- κ B/Rel family of transcription factors is involved in various aspects of cellular proliferation and oncogenesis. However, the precise contribution of NF- κ B in promoting growth advantages is poorly defined. Although a number of cytokines and gene products depend on their ability to activate NF- κ B to induce cell proliferation, these agents may have other properties which contribute to this process. The oncogenicity of v-Rel and the role of NF κ B2 in leukemogenesis remain the clearest evidence that Rel proteins can participate in oncogenesis.

4. Regulation of HIV-1 Transcription

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The human immunodeficiency virus (HIV-1) is the causative agent of acquired immunodeficiency syndrome (AIDS). The clinical manifestation of HIV-1 infection is a depletion of CD4+ T cells and the gradual collapse of the host immune system. The tropism and infectivity of the virus is determined by the interaction between the envelope gp120 and CD4 molecules expressed on T cells and myeloid cells. The intracellular efficiency of HIV-1 gene expression and replication is due in part to the ability of HIV-1 to utilize host signaling pathways to mediate its own transcriptional

regulation. In this regard, the NF- κ B/Rel pathway plays a central role in HIV-1 long terminal repeat (LTR) driven transcription. NF- κ B synergizes with the HIV-1 Tat protein and cooperates with other transcription factors to strongly activate transcription. In chapter VI, the ability of I κ B α mutants to repress NF- κ B-dependent HIV-1 transcription and replication is examined. Consequently, the contribution of NF- κ B and HIV-1 Tat protein to HIV-1 LTR transcription is briefly reviewed below.

4.1 NF-kB-Tat regulation of the HIV-1 LTR

The HIV-1 LTR contains two adjacent high affinity xB binding sites in the enhancer region of its LTR (-109 to -79) (202). Transient transfection studies using HIV LTR or HIV enhancer reporter constructs demonstrate that HIV gene expression increases upon induction of NF-xB DNA binding activity with stimulators such as TNF- α and IL-1 (reviewed in 12, 238, 288). The effect of the different NF-xB subunit combinations on transactivation of the HIV-1 enhancer, has been intensively studied. The studies reveal that heterodimers composed of NFxB2/p52 and ReIA subunits have a higher affinity for the HIV-1 enhancer than the prototypical NFxB1/p50·ReIA heterodimer (77). Interestingly, nuclear translocation of c-Rel, which occurs late after translocation of NFxB1/p50 and ReIA, was shown to compete for the binding of ReIA to the HIV-1 enhancer and to repress transcription (76).

Experiments using HIV LTR reporter constructs in lymphoid cells showed that mutation of NF- κ B motifs reduced gene expression in the presence and absence of the HIV-1 Tat protein (3,10,28,29,89,111,114,173,202,278). The 15 kDa Tat protein enhances LTR-derived gene expression and is required for high-level expression of all viral genes (reviewed in 65). Tat acts via physical association with a stem-loop KNA structure, the transactivation response (TAR) sequence (94,239,240), present in the 5' end of all nascent viral mRNA species. TAR RNA is formed by nucleotides +1 and +59 and the core secondary stem-loop structure (nucleotides +18 to +44) is critical for Tat-

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TAR interactions and for Tat-mediated transactivation. The bulge region in TAR (+22 to +24) serves as the primary binding site for Tat, and the loop sequences (+30 to +35) also contribute to this interaction (239,240,261). TAR RNA essentially serves as an anchor for Tat and certain cellular factors (TRP-185 and p68) (179,264) to facilitate interactions with promoter elements such as TATA, Sp1 and the HIV-1 enhancer (29,139). Deletion of NF- κ B and Sp1 binding sites from the HIV-1 promoter abrogates Tat-mediated transactivation, suggesting that once brought into the vicinity of the promoter, Tat interacts with transcription factors bound at the NF- κ B/Sp1 region to stimulate transactivate independently of TAR RNA, since TAR defective viruses are replication competent but still require Tat. In each case, the Tat-responsive element maps to the NF- κ B sites within the HIV enhancer (3,13,278).

Specific Research Aims

The global objective of this research was to understand the mechanisms by which the inhibitor $I\kappa B\alpha$ regulates the NF- κB family of transcription factors. The first aim was to examine the effect of ectopic $I\kappa B$ expression in the murine fibroblastic cell line NIH 3T3. The results illustrate the central role of $I\kappa B\alpha$ in NF- κB regulation and cell growth. This prompted us to investigate the mechanisms controlling $I\kappa B\alpha$ degradation. The second objective was to determine the functional domains of $I\kappa B\alpha$ involved in inducer-mediated phosphorylation and degradation. The results obtained in reaching this objective, together with results obtained by others, led to the design of mutants that are predicted to repress NF- κB activity in stimulated cells. The third aim was to determine the dominant negative phenotype of these $I\kappa B\alpha$ mutants in the context of the HIV-1 infection. This work demonstrated that HIV-1 replication can be repressed by the presence of $I\kappa B\alpha$ mutants resistant to inducer-mediated degradation. These studies have permitted the design of a structure-function model to explain the activity of $I\kappa B\alpha$.

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Chapter II

Materials and Methods

1 Antisera

The preparation of rabbit antisera was performed by Anne Roulston and is described in ref (216) and (227). The sequence recognized by the different NF-xB antibodies is represented in Figure 8. AR28 was raised against a peptide corresponding to the Cterminal aa 537 to 550 of human RelA(p65), and recognizes both human and murine RelA(p65). AR22 was raised against a peptide corresponding to the C-terminal aa 573 to 587 of human c-Rel and recognizes human but not murine c-Rel. AR27 was raised against a peptide corresponding to the N-terminal as 2 to 15 of human NF κ B1/p105 and recognizes both human NFkB1/p105 and NFkB1/p50 but not their murine homologues. AR25 was raised against a peptide corresponding to the C-terminal aa 955 to 969 of human NFxB1/p105 and recognizes both human and murine NFxB1/p105 and 1xBy. AR43 was raised against a peptide (aa 2-17) in the N-terminus of NFkB2/p100 and recognize both human and murine NF κ B2/p100 and NF κ B2/p52. AR40 was raised against a peptide (aa 928-940) at the C-terminus of NFxB2/p100 and recognizes both human and murine NFxB2/p100 (158). AR20 was raised against a peptide corresponding to the N-terminal aa 2 to 16 of $I\kappa B\alpha$ and recognizes both murine and human IxB α (236). For some experiments, AR20 was affinity purified on Affi-Gel 15 column (Bio-Rad) coupled to a peptide corresponding to aa 2 to 16 of human $l\kappa B\alpha$. according to manufacturers instruction.

sc-114 and sc-70 are commercial affinity purified rabbit antisera (Santa Cruz Biotech): sc-114 was raised against a peptide encompassing part of the nuclear localization sequence of NF κ B1/p50 (aa 350 to 363) and recognizes human and murine NF κ B1/p50; sc-70 was raised against a peptide corresponding to aa 152 to 176 within the N-terminal domain of the predicted sequence of murine c-Rel. sc-70 recognizes both mouse and human c-Rel proteins. The monoclonal antibody MAD 10B directed against an epitope located between aa 21 and aa 48 of I κ B α was kindly provided by Dr. R.T. Hay (134). Anti-casein kinase II rabbit antibody (Upstate Biotechnology Inc.) was



Figure 8. NF- κ B specific antisera. The primary structure of the various NF- κ B subunits is represented schematically (216, 227). Black boxes indicate the sequence recognized by the antibody. NLS: nuclear localization sequence; Rel homology domain ; Antipology antipology antipology is antipology. The primary structure of the various NF- κ B sequence; Rel glycine rich region.

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raised against a synthetic peptide corresponding to the residues 70 to 91 of the alpha catalytic subunit of human casein kinase II. Monoclonal murine anti-tubulin and antiactin antibodies were obtained from Boehringer Mannheim and ICN Biomedicals respectively. Human sera from an HIV-1 seropositive individual was kindly provided by Dr. Mark Wainberg.

2 Recombinant DNA

2.1 Reporter gene constructs

The construct ptzIIICAT contains the entire HIV-1 LTR linked to the chloramphenicol acetyl transferase (CAT) gene. The plasmid ptzIIICAT and the mutated derived plasmids -109/-79, III Δ 23 and III Δ A were a kind gift from Dr. Eric Cohen (Figure 9). The -109/-79 plasmid contains a mutated HIV-1 LTR lacking the NF- κ B sites. The III Δ 23 construct is deleted for the upstream modulatory sequences (upstream of -167) and the plasmid III Δ A is deleted for all of the LTR sequences upstream of -57. Δ B and B-ACU constructs are derived from the HIV LTR CAT pU3RIII construct but encode a mutated and non functional TAR element (239). The CAT reporter constructs pHIV-CAT and pHIV(mut)-CAT contain a single copy of the HIV-1 enhancer (-105 to -80) or a mutated copy of the enhancer linked to the basal SV40 promoter, respectively (161) (Figure 9). The construct pSV1-CAT contains the reporter CAT gene under the control of a basal SV40 promoter (105).

2.2 PMV-7 based retroviral vectors

The retroviral pMV-7 vector contains the neo resistance gene under the control of the herpes virus simplex virus-1 (HSV-1) thymidine kinase (TK) promoter, together flanked by the 5' and 3' murine moloney leukemia virus LTR (146). Human I κ B α (112) and I κ B γ (aa 366 to 969 of human NF κ B1/p105) cDNAs were subcloned into the EcoR1 site of pMV-7 (downstream of the 5' LTR) in both the sense and antisense orientations. The

Figure 9. Reporter gene constructs. The U3 and R region of HIV-1 LTR are subdivided into the modulatory and negative regulatory elements, the enhancer (including two NF- κ B sites), the core promoter and the TAR elements to which viral and cellular factors bind. HIV CAT reporter plasmids contain wild type or mutated versions of the HIV LTR, linked to the CAT gene: all encode the TAR element. The HIV enhancer plasmids contain a minimal SV40 promoter fused to the NF- κ B sites from the HIV LTR.

HIV LTR CAT plasmids



HIV enhancer CAT plasmids

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TAR RNA

cDNA clone encoding p105 was obtained from A. Israel, Institut Pasteur, and the region corresponding to $I\kappa B\gamma$ nucleotides 1284 to 3090 (encoding aa 366 to 968 of p105) was obtained by polymerase chain reaction of the NF κ B1/p105 cDNA with specific primers corresponding to nucleotides 1280 to 1299 (5'-AGCTGGAATTCTCATGCCCAA TTTTTCGG-3') and 3187 to 3199 (5'-TGCAGGAATTCCGGCCGGCTCTC-3') (144).

2.3 IkB α mutagenesis

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C-terminal deletion mutants $I\kappa B\alpha(\Delta 1)$, $I\kappa B\alpha(\Delta 2)$, $I\kappa B\alpha(\Delta 3)$ and $I\kappa B\alpha(\Delta 4)$ were generated by Dr. Rongtuan Lin by inserting an artificial stop codon in the human $I\kappa B\alpha$ gene at the amino acid position 261, 269, 288 and 296, respectively. cDNAs encoding $I\kappa B\alpha$ carboxyl terminal deletion mutations were generated by 25 cycles of PCR amplification. DNA oligonucleotide primers were synthesized using an Applied Biosystems DNA/RNA synthesizer. The amino-terminal primer was synthesized with an *Eco*RI restriction enzyme site and the carboxy-terminal primers were synthesized with *Eco*RV restriction enzyme sites at their ends.

The point mutations of $I \ltimes B \alpha$ were introduced by overlap PCR mutagenesis using Pfu DNA polymerase (122). The amino-terminal and carboxyl terminal primers were synthesized with *Eco*RI restriction enzyme sites at their ends. Mutations were confirmed by sequencing. $I \ltimes B \alpha(DM)$ is a full length human $I \ltimes B \alpha$ in which serine 283 and threonine 291 were substituted for alanine residues. In $I \ltimes B \alpha(3C)$. serine 283, threonine 291 and threonine 299 were all substituted for alanine residues. In $I \ltimes B \alpha(2N)$ mutant the serine 32 and 36 were both substituted to alanine and in mutant $I \ltimes B \alpha(2N +$ 3C), serine 32, 36, 283 and threonine 291 and 299 were all substituted to alanine. Substitution of only serine 32 or serine 36 to alanine was introduced in mutants S32 and S36 respectively. The cDNA encoding $I \ltimes B \alpha(2N)$ was amplified by PCR using the set of primers used to generate $I \ltimes B \alpha(\Delta I)$. The resulting cDNA- $I \ltimes B \alpha(2N \Delta I)$ -has an alanine at position serine 32 and serine 36 and is deleted for the 56 carboxyl terminal amino acids.

2.4 Constitutive mammalian expression vectors

Wild type and mutated human IxBα cDNAs, as well as the p50/65 fusion gene, were inserted downstream of the Simian Virus-40 (SV40) promoter in the pSVK3 mammalian expression vector (Pharmacia). The p50/65 gene is a fusion of the amino-terminal region of human p105 (coding for aa 1 to 502) and the carboxy-terminal of human p65 (coding form aa 397 to 550) (166). CMV-p65 is a cytomegalovirus (CMV) promoter-driven expression plasmid encoding RelA(p65) (216). The plasmids expressing wild type and R52Q, R53Q mutants of HIV-1 Tat, as well as the plasmid containing the HIV-1 proviral DNA (pSVC21 BH10) were previously described (58.70).

2.5 Tetracycline regulated expression vector system

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Plasmids pREP-4 and pREP-9 (InVitrogen) encode the Epstein-Barr virus (EBV) protein EBNA-1 and contain the EBV origin of replication OriP. These plasmids can therefore be maintained in mammalian cells in an episomal state. pREP-4 and pREP-9 confer hygromycin and neomycin resistance, respectively. A cDNA encoding the hybrid transactivator tTA (106), a fusion protein between the tetracycline repressor and the HSV VP16 transactivator, was inserted into the *Hind* III - *Bam*H I sites of the pREP-4 plasmid. In the resulting plasmid (pREP-4-tTA), tTA transcription is controlled by the Rous Sarcoma Virus (RSV) promoter, and tTA activity is inhibited by tetracycline (106), at concentrations ranging from 0.1 to 1.0 μ g/ml (Figure 10).

Wild type and mutant $I\kappa B\alpha$ cDNA were cloned downstream of the tetracycline responsive promoter CMV_t which consists of a minimal promoter sequence derived from the human CMV IE promoter fused to seven copies of *tet* operator sequences (106). These CMV_t-I κ B α cassettes were inserted in the pREP-9 plasmid between the *Xho* I and *Bam*H I sites, thus replacing the RSV promoter. In the resulting pREP-9-



Figure 10. The tetracycline responsive control of $I\kappa B\alpha$ expression. Plasmid pREP-tTA expresses the tetracycline sensitive transactivator tTA. In the pREP-CMVt-I $\kappa B\alpha$ plasmid, $I\kappa B\alpha$ transcription is driven by the CMVt promoter which is transactivated by tTA. The presence of the OriP origin of replication and expression of EBNA-1, allow pREP-tTA and pREP-CMVt-I $\kappa B\alpha$ plasmids to be maintained in cells in an episomal state.

IxB α plasmids, transcription of wild type or mutant IxB α gene is transactivated by the transactivator tTA (Figure 10).

2.6 Bacterial expression vectors

The plasmid for expression of GST-Np65 fusion proteins was constructed as follows: p65-Bluescript (a kind gift from Dr. Craig Rosen) was digested with *Sma-1* and *Taq-1*; the 1 kb fragment liberated, encoding RelA(p65) aa 1 to 329 (mutant Np65), was filled in with Klenow polymerase and inserted into the *EcoR-1* site (also filled in with Klenow enzyme) of pGEX 2T plasmid. For plasmids encoding recombinant wild-type or mutant IxB α -GST fusion proteins. IxB α cDNAs were digested with *Eco*RI and *Eco*RV, and inserted into *Eco*RI/*Tih*III1 sites (*Tth*III1 site was filled in with Klenow) of pGEX-2T (Pharmacia Biotech Inc.).

3 Cell lines

3.1 Cell lines and maintenance.

Cells were cultured at 37°C in a CO₂ incubator (5% CO₂). The immortalized murine fibroblasts cell line NIH 3T3, was maintained in Dulbecco's modified Eagle's medium (D-MEM) (Gibco, Life Technologies Inc.) supplemented with 10% heat inactivated fetal bovine serum (Hyclone). The CD4+/CD8- human immature T helper lymphocytes cell line Jurkat, was maintained in RPMI 1640 (Gibco, Life Technologies Inc.) supplemented with 10% heat inactivated calf serum (Hyclone). The embryonal carcinoma cell line N-Tera-2 (38,310) and the canine fibroblast packaging cell line DAMP (88) were maintained in D-MEM plus 10% heat inactivated fetal bovine serum (Hyclone). All culture media were supplemented with 2 mM L-glutamine and 20 μ g/ml gentamycin (Schering Canada).
3.2 NIH 3T3 cells transduced with PMV-7 derived retroviral vectors

Retroviral vectors were introduced into the amphotropic packaging cell line DAMP by electroporation (88). Briefly, 6 x 10⁶ DAMP cells were resuspended in 0.8 ml phosphate buffered saline (PBS) containing 40 μ g of pMV-7 retroviral vector DNA. Electroporation was performed using the Gene Pulser system (Bio-Rad) at a tension of 450 volts and a capacitance of 250 μ F. Cells were selected in D-MEM supplemented with 10% fetal bovine serum (heat inactivated) and G418 (500 μ g/ml) for two weeks. Equal numbers of virus-producing DAMP cells were grown in exponential phase in the absence of G418 for two days. Culture supernatants were collected, passed through a 0.22 μ m filter, and were used to infect subconfluent NIH 3T3 cells for 48h. Infected cells were then selected in D-MEM plus 10% calf serum and G418 (500 μ g/ml) for two weeks. Individual colonies were isolated and maintained individually.

3.3 NIH 3T3 cells stably transfected with pSVK3 derived plasmids.

pSVK3- $I\kappa B\alpha$, pSVK3- $I\kappa B\alpha(2N\Delta 1)$ or pSVK3 plasmids (10 µg) were introduced into NIH 3T3 together with pRSVneo plasmid (0.1µg) by lipofection (LipofectAmine, Gibco, Life Technologies Inc.) according to the manufacturer's instructions. G418 resistant colonies were isolated as described above.

3.4 NIH 3T3 cells stably transfected with tetracycline reponsive expression vectors. Plasmid pREP4-tTA was introduced in NIH 3T3 cells by lipofection (Lipofectamine, Gibco BRL) according to the manufacturer's instructions. Cells were selected beginning at 48h in D-MEM containing 10% calf serum and 300 μ g/ml Hygromycin B (Boeringer Mannheim). Resistant cells carrying the pREP4-tTA plasmid (tTA-3T3 cells) were then transfected with the various pREP9-CMV_t-IxB α plasmids. Cells were selected and maintained in D-MEM media containing 10% calf serum, 300 μ g/ml Hygromycin B and 400 μ g/ml G418 (Gibco BRL). Colonies of resistant cells

(tTA-I κ B α) were expanded individually or pooled together to create polyclonal population. NIH 3T3 derived lines inducibly expressing wild type I κ B α , or the mutants I κ B $\alpha(\Delta 1)$, I κ B $\alpha(\Delta 2)$, I κ B $\alpha(\Delta 3)$, I κ B $\alpha(\Delta 4)$, I κ B $\alpha(2N)$, I κ B $\alpha(2N + 3C)$ or I κ B $\alpha(3C)$ were generated. At all times, cell lines were maintained in presence of tetracyline (1 µg/ml) to repress exogenous I κ B α expression. Before each experiments, cells to be analyzed were grown in absence of tetracycline for 72h to allow exogenous I κ B α expression.

4 Characterization of the growth phenotype

4.1 Saturation densities

Saturation density was determined in cell cultures 4 days after confluency was achieved. Numbers are the average of three separate experiments performed on three or four cell lines, each initially plated at a density of 1 x 10^5 cells/35 mm dish. Cells were trypsinyzed and counted in Isoton II solution (Coulter Electronics of Canada) using an electronic cells counter (Coulter Electronics of Canada).

4.2 Soft agar growth assay

For the soft agar assay, cells (1 x 10^5) were suspended in a 0.35% agar solution in D-MEM containing 20% FBS and overlaid onto 0.5% agar solution in D-MEM containing 20% FBS in 100 mm plates. Cells grown in soft agar were counted 20 days after plating, and cloning efficiency was determined as the number of colonies x 100 divided by the number of cells plated (average of two experiments).

4.3 Tumorigenicity in nude mice

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To assay for tumorigenicity, cells (1×10^6) suspended in 100 µl of PBS were injected subcutaneously into the left side hind limb of 4 to 8 week old athymic mice (CD1 nu/nu; Charles River). The experiment was performed using two independently isolated cell clones; three mice per clone were injected. The time required to produce tumors of at least 5 mm diameter was considered the latency period. Mice were inspected for 60 days.

5 Purification and phosphorylation of recombinant proteins.

For the production of Glutathione-S-transferase fusion IxBα proteins, the E. coli strain DH5α (Life Technologies, Inc.) was transformed with pGEX2T/IxBα plasmids. After induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) (Pharmacia Biotech Inc.) at 37°C, bacteria were lysed in PBS containing 1% Triton X-100. Lysates were incubated with glutathione-Sepharose beads (Pharmacia Biotech Inc.) for 20 min at room temperature. After washing three times with PBS, the fusion protein was enzymatically cleaved with thrombin (Sigma) in 50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 2.5 mM CaCl₂ for 2 to 4h at room temperature. The GST moeity remained on the column and the protein of interest was eluted. Alternatively, the whole GST fusion protein were eluted from the column with 15 mM glutathione. Phosphorylation of IxBα recombinant proteins (2 ng) by casein kinase II (CKII) was performed for 30 min at 30^αC with 5 units of recombinant CKII enzyme (New England Biolab) in a buffer containing 25 mM Tris-HCl, pH 7.5; 50 mM KCl; 1 mM MnCl₂; 1 mM MgCl₂; 10 mM ATP.

6 **Protein detection methods**

6.1 Immunoblotting

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For preparation of whole cell protein extracts, cells were washed with phosphate buffer saline (PBS) and collected in TEN buffer: 40 mM Tris HCl, pH 7.5; 1 mM [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid] (EDTA), pH 8.0; 150 mM NaCl; 2 mM phenylmethylsulfonyl fluoride (PMSF). Cells were then lysed in WBL buffer: 10 mM Tris HCl pH 8.0; 60 mM KCl; 1 mM EDTA; 1 mM dithiothreitol (DTT); 0.5% Nodidet P-40; 0.5 mM PMSF; 0.01 µg/µl Leupeptin; 0.01 µg/µl Pepstatin; 0.01 µg/µl

Aprotinin. Insoluble cellular debris were removed by centrifugation at 10,000 g for 5 min at 4°C. The protein concentration of extracts was estimated using the Bradford protein assay from Bio-Rad.

For preparation of immunoblots, equivalent amount of protein from different samples were boiled in 10 μ l of 2x sample buffer and were subjected to 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (310). Resolved proteins were transferred to a nitrocellulose membrane (Amersham), and stained with 5% Ponceau red; 10% acetic acid to verify that equivalent amount of protein was used from each sample. The nitrocellulose blot was incubated with protein specific antiserum diluted in 5% milk in PBS, for 1-16h. After four washes with PBS for 10 min, membranes were reacted with peroxidase-conjugated anti-immunoglobulin antibody (Amersham) for 1h. After four 10 min washes with PBS, detection of antigen-antibody complexes was performed using the ECL western blotting detection reagents (Amersham) according to the manufacturer's instructions.

6.2 Measuring the half life of IxBα

tTA-I κ B α expressing cells cultured in tetracycline free D-MEM media supplemented with 10% calf serum, were stimulated with 5 ng/ml TNF- α (Gibco BRL) or 10 µg/ml LPS (Sigma), either with or without addition of 50 µg/ml cycloheximide. In some experiments, cells were pre-treated for 1h with either 100 µM calpain inhibitor I (ICN), 40 µM MG132 proteasome inhibitor (kindly supplied by MyoGenics Inc.) (213) or an equivalent volume (8 µl/ml) of their respective solvent (ethanol and dimethyl formamide respectively) as a control. Whole cell extracts were prepared and I κ B α was detected by immunoblotting using I κ B α monoclonal antibody MAD 10B (134) or affinity purified AR20 antibody (216).

6.3 Immunoprecipitation

Cells were washed with PBS and labeled for 60 min in methionine free RPMI 1640 containing 400 μ Ci/ml Tran³⁵S Label (Amersham). Cells were collected in 40 mM Tris HCl, pH 7.5; 1 mM EDTA, pH 8.0; 150 mM NaCl; 2 mM PMSF (TEN buffer) and lysed in 20 mM Tris HCl, pH 7.5; 200 mM NaCl; 0.5% Nodinet P-40; 2 mM PMSF (TNT buffer). Cell lysates (300 µg) were incubated with 10 µl of IkBa antibody (AR20) or RelA(p65) antibody (AR28) and 30 µl of protein A-Sepharose beads (Pharmacia) for 3h at 4°C. Beads were washed five times with TNT buffer and the immunoprecipitates were eluted by boiling the beads for 3 min in 1% SDS; 0.5% β-mercaptoethanol. The eluate was diluted with one volume of TNT buffer and incubated overnight at 4°C with 10 µl of IkBa or RelA(p65) antibody. Beads were again washed five times with TNT buffer and the immunoprecipitate was eluted by boiling the beads for 3 min in 1% SDS; 0.5% β-mercaptoethanol. The eluate was diluted with one volume of TNT buffer and incubated overnight at 4°C with 10 µl of IkBa or RelA(p65) antibody. Beads were again washed five times with TNT buffer and the immunoprecipitate was eluted by boiling the beads for 3 min in SDS sample buffer. Eluted proteins were electrophoresed on 10% SDS PAGE and detected by autoradiography. The specificity of IkBa antibody recognition was confirmed by competition with IkBa peptide (aa 2-16).

6.4 Detection of RelA·IxBα complexes.

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For detection of $1 \times B\alpha$ bound ReIA, SVK3 clone no.3 and p50/65 clone no.7 cells were washed with PBS and lysed in TNT buffer (200 mM NaCl. 20 mM Tris-HCl pH 7.5, 0.5 % Nodinet P-40). Cell lysates (300µg) were incubated with 10 µl of AR20 antibody and 30 µl of protein A-Sepharose beads (Pharmacia) for three hours at 4°C. The specificity of the antibody was confirmed by the competitive addition of 5 µg of $1\times B\alpha$ peptide (aa 2-15). Beads were washed five times with 750 µl TNT buffer and the $1\times B\alpha$ immunoprecipitate was eluted by incubating the beads with 10 µl of 1 µg/µl $1\times B\alpha$ peptide (aa 2-15) in TNT buffer for 16h at 4°C. The presence of RelA(p65) and p50/65 in the eluted $1\times B\alpha$ immunoprecipitate was detected by immunoblotting using RelA(p65) antibody (AR28).

For detection of RelA bound $I\kappa B\alpha$, NIH 3T3 cells were transfected by lipofection (LipofectAmine, Promega) with 10 µg of pSVK3/I κ B α plasmids or pSVK3 plasmid as a control. Forty eight hours after transfection, cells were washed with PBS, collected in TEN buffer and lysed in TNT buffer. For each sample, 300 µg of protein extract was incubated for 16h at 4°C with 10 µl of RelA(p65) (AR28) antibody (or $I\kappa B\alpha$ (AR20) antibody as a control) and 30 µl protein-A-sepharose. Beads were washed five times with 750 µl TNT buffer. RelA immunoprecipitates were eluted by boiling the beads for 3 min. in 1% SDS: 0.5% β-mercaptoethanol. Presence of $I\kappa B\alpha$ in the immunoprecipitates was detected by immunoblotting using MAD 10B monoclonal $I\kappa B\alpha$ antibody.

6.5 Detection of hyperphosphorylated IkBa in vivo

Hyperphosphorylation retards $I\kappa B\alpha$ migration on SDS PAGE. The following protocol maximizes the resolution of hyperphosphorylated $I\kappa B\alpha$. NIH 3T3 cells overexpressing wild type or mutant $I\kappa B\alpha$ were treated for 0, 1, 5 or 60 min with 5 ng/ml TNF- α and whole cell extracts were prepared. Fifty micrograms of protein extracts were electrophoresed on a 7% to 13% gradient SDS polyacryacrylamide gel. Proteins were transferred on nitrocellulose and $I\kappa B\alpha$ was detected using affinity purified $I\kappa B\alpha$ antibody AR20. Hyperphosphorylated $I\kappa B\alpha$ migrates more slowly and appears as a distinct band sensitive to phosphatase treatment. Phosphatase treatment of cell extracts prior to electrophoresis was performed using 8 units of calf intestinal phosphatase (Promega) in 40 µl of 0.05 M Tris HCl, pH 9.0; 1 mM MgCl₂; 0.1 mM ZnCl₂; 1 mM spermidine for 15 min at 37°C. Specific inhibition of the phosphatase activity of CIP was achieved by adding 10 mM Na₃VO₄ and 50 mM NaF to the reaction. Reactions were stopped by the addition of 10 µl of 5x sample buffer and 3 min incubation at 98°C. In the following protocol, the phosphorylation state of I $\kappa B\alpha$ is directly measured by quantifying the radiactivity of I $\kappa B\alpha$ in cells labeled with ³²P orthophosphate. NIH 3T3

cells expressing wild type or mutant IkBa proteins were washed twice with serum and phosphate free D-MEM and then incubated in phosphate free D-MEM containing 2.5% dialyzed calf serum for 45 min; the medium was then replaced with fresh phosphate free D-MEM supplemented with 10% dialyzed calf serum and 1 mCi/ml of ³²Porthophosphate. As a control, cells were also labeled with ³⁵S methionine. For ³⁵S methionine labeling, cells were washed twice with serum and once with methionine free D-MEM and then incubated in methionine free D-MEM containing 2.5% dialyzed calf serum for 45 min.; the medium was then replaced with fresh methionine free D-MEM supplemented with 10% dialyzed calf serum and 300 µCi/ml of ³⁵S-methionine (ICN). The cells were labeled at 37°C with shaking during 6h for ³²P orthophosphate labeling or during 4h for 35 S-methionine labeling. For TNF- α induction, cells were pre-treated with 100 μ M of calpain inhibitor I for 1h, then stimulated with TNF- α (5 ng/ml) for 30 min. The labeled cells were washed with PBS and lysed in TNT buffer. Cell lysates (100 µg) were incubated with 10 µl of IkBa antibody (AR20) and 30 µl of protein A-Sepharose beads (Pharmacia) at 4°C overnight. Beads were washed five times with TNT buffer and the immunoprecipitate was eluted by boiling the beads 3 min. in 30 μ l of 0.5% 2-mercaptoethanol and 1% SDS. The eluate was then diluted 1:2 with TNT buffer and incubated with 10 μ l of IkB α antibody and 30 μ l of protein A-Sepharose beads for 6h at 4°C. Beads were again washed five times with TNT buffer and the immunoprecipitate was eluted by boiling the beads 3 min. in SDS sample buffer. Eluted proteins were electrophoresed on 10% SDS PAGE. Proteins were transferred to nitrocellulose membrane and detected by autoradiography.

6.6 GST affinity chromatography

Recombinant IxB α proteins (10 ng) cleaved with thrombin to remove the GST moiety, were incubated for 90 min. at 4°C with 25 μ l glutathione agarose beads coated with GST-Np65 fusion protein or GST alone as a control. The beads were washed 3 times in 20 mM Hepes pH 7.9; 150 mM NaCl; 10% glycerol; 0.1% Triton (HNTG buffer). GST-Np65 and GST bound IxB α were eluted with 50 mM Tris-HCl, pH 8.0; 15 mM glutathione. One third of the eluate was boiled in SDS sample buffer and was analyzed by immunoblotting using affinity purified IxB α antibody (AR20).

7 Electromobility shift assay

7.1 Oligonucleotide probes

Complementary oligonucleotides containing two NF- κ B sites derived from the human immunodeficiency virus type 1 (HIV-1) enhancer or 2 copies of the PRDII domain of the IFN- β promoter were synthesized using an Applied Biosystems DNA/RNA synthesizer (236). Complementary oligonucleotides containing mutated NF- κ B sites were also synthesized (236). Complementary oligonucleotides were mixed together in TNE buffer (10 mM Tris-HCl, pH 7.5; 100 mM NaCl; 1 mM EDTA), heated for 10 min. at 85°C and slowly cooled down to room temperature to maximize annealing. Double stranded oligonucleotides were purified on a TBE (50 mM Tris-HCl, pH 8.0; 50 mM boric acid; 1 mM EDTA) 12% polyacrylamide gel (19:1 cross link) and end labeled with [γ -³²P] ATP (ICN) using T4 polynucleotide kinase (Pharmacia). Unincorporated nucleotides and salts were removed on G-25 Sephadex column (Pharmacia).

HIV-1 enhancer probe:	5 '-aGGGACTTTCCgctGGGACTTTCC-3 '
HIV-1 enhancer mutant probe:	5'-acatggtTTCCgctcatggtTTCC-3'
PRDII (P2) probe:	5'-GGGAAATTCC···GGGAAATTCC-3'
PRDII (P2) mutant probe:	5'-actAAATTCC···actAAATTCC-3'
consensus NF-κB sequence:	5 '-GGGRNNYYCC-3 '

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7.2 Extract preparation and analysis

For nuclear extracts preparation (211), 1 x 10⁷ cells were washed once with cold PBS and once with buffer A (10 mM Hepes, pH 7.9; 1.5 mM MgCl₂; 10 mM KCl; 0.5 mM DTT: 0.5 mM PMSF). Plasma membranes were disrupted by resuspending cells in 20 µl of 0.1% Nodinet P-40 in buffer A Cells were incubated for 10 min on ice and centrifuged at 10,000g for 10 min at 4°C. The supernatant (cytoplasmic extract) was collected and set aside. The nuclei pellet was resuspended and lysed in 15 µl buffer B (20 mM Hepes, pH 7.9; 25% glycerol; 420 mM NaCl; 1.5 mM MgCl₂; 0.2 mM EDTA; 0.5 mM DTT: 0.5 mM PMSF: 0.5 mM spermidine: 0.15 mM spermine: 0.01 µg pepstatin, 0.01 µg leupeptin; 0.01 µg aprotinin). Nuclei were incubated 15 min on ice and centrifuged at 10,000g for 15 min at 4°C. The supernatant (nuclear extract) was diluted with 75 µl of buffer C (20 mM Hepes, pH 7.9; 20% glycerol; 0.2 mM EDTA; 50 mM KCl: 0.5 mM DTT; 0.5 mM PMSF). Protein concentration of the extract was measured using the Bradford protein assay from Bio-Rad.

Five micrograms of nuclear extracts were pre-incubated with the non-specific DNA competitor poly(dI:dC) (5 µg, Pharmacia) for 10 min. at room temperature. ³²Pradiolabeled probe was added and the mixture was incubated for 20 min longer at room temperature. DNA-protein complexes were resolved on a 5% non-denaturing polyacrylamide (60:1 cross-link)/Tris glycine (25 mM Tris-HCl; 195 mM glycine) gel and were visualized by autoradiography. To demonstrate the specificity of the protein-DNA complex formation, 200 fold molar excess of either mutated or wild type unlabeled probe was added to the extract 10 min before the addition of ³²P-radiolabeled probe. For supershift assays, nuclear extracts were pre-incubated with NF-kB subunitspecific antisera (1 µl) for 10 min at room temperature, prior to the addition of poly (dI:dC) and radiolabeled probe. To demonstrate the specificity of antibody recognition. $2 \mu g$ of the peptide ($2 \mu g$) used to raise the antibody was added to the extract prior to the addition of antibody. To evaluate the DNA binding inhibitory activity of $I\kappa B\alpha$ or the

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various I κ B α mutants, 2 ng of recombinant I κ B α or mutant I κ B α was added to the extract 10 min before the ³²P-radiolabeled probe was added. Similarly, CKII phosphorylated recombinant I κ B α proteins (2 ng) were tested for their ability to inhibit NF- κ B DNA binding activity.

8 Reporter gene assay and transfections

8.1 Transfection of NIH 3T3 cells by lipofection.

Plasmids pSV1 CAT, pHIV CAT and pHIV-mut CAT (10 μ g) were introduced into NIH 3T3 cells by lipofection (LipofectAmine, Gibco BRL) according to the manufacturer's instructions. Media was replaced with D-MEM supplemented with 0.2% heat inactivated calf serum 12h after transfection and cells were harvested 36h later.

8.2 Transfection of Jurkat cells by the DEAE dextran method

Jurkat cells were transiently transfected by DEAE-dextran method (156). The precipitated DNAs (0.5 to 8 μ g), representing either HIV-LTR CAT reporter plasmids and pSVexTat plasmids (wtTat or R52Q,R53Q Tat mutant) (70) were resuspended in TS solution (25 mM Tris HCL, pH 7.4; 137 mM NaCl; 5 mM KCl; 0.6 mM Na₂HPO4; 0.7 mM CaCl₂; 0.5 mM MgCl₂). After resuspension, 0.05 mg of DEAE-Dextran (Pharmacia) was added. For each transfection, 1x10⁷ cells in exponential phase were washed once in TS, resuspended with the DNA solution and incubated at room temperature for 20 min. Cells were then incubated at 37°C for 30 min. in 10 ml of medium containing 10% serum and 0.1 mM chloroquine (Sigma Chemical Co.), after transfection, cells were induced with 5 ng/ml TNF- α (Boeringer Mannheim). At 16h after induction, cells were harvested.

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8.3 Transfection of N-Tera-2 cells by the calcium phosphate method

Using the calcium phosphate method (167), N-Tera-2 cells were co-transfected with pHIV-CAT reporter plasmid (3 μ g) along with CMV-p65 plasmid (3 μ g) and various pSVK3 plasmids (Pharmacia) encoding wild type or mutated I κ B α (9 μ g). In pHIV-CAT plasmid, the chloramphenicol acetyl transferase gene is under the control of a minimal SV40 promoter fused to one copy of the HIV-1 enhancer. At 30h after transfection, cells were stimulated with 25 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma). Cells were harvested 48h after transfection.

8.4 CAT assay

Cells, were lysed in 0.25 M Tris-HCl, pH 7.5 by successive freezing and thawing. Cellular debris were collected by centrifugation at 10,000g for 10 min at 4°C. The protein concentration of the extracts was measured using the Bradford protein assay from Bio-Rad. Equivalent amount of protein extract (from 10 μ g to 300 μ g depending on the experiment) were assayed in 150 μ l of 1 mM acetyl CoA; 0.25 M Tris-HCl, pH 7.5 in presence of 0.5 μ Ci of [¹⁴C]chloramphenicol. The reaction was incubated at 37°C for 30 min to 4h and [¹⁴C]chloramphenicol was extracted in ethyl acetate. Acetylated chloramphenicol was resolved from unacetylated chloramphenicol by ascending thin layer chromatography in 95% chloroform; 5% methanol. The percent acetylation was measured using the Bio-Rad Gelscan Phosphoimager and the Molecular Analyst (Bio-Rad) software program.

9 RNA detection

9.1 Extraction of RNA

Polyadenylated RNA was isolated using the Fast Track isolation kit (InVitrogen). Total RNA was isolated using RNeasy kit from Qiagen according to manufacturer's instructions or by the guanidium-isothyocyanate method (55). For RNA isolation using

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the guanidium-isothyocyanate method, cells ($1 \ge 10^6$ to $1 \ge 10^7$) were lysed in 400 µl of solution D (25 mM sodium citrate, pH 7.0; 4M guanidine isothiocyanate; 0.5% sarcosyl, and 100 mM 2-mercaptoethanol). The viscous lysate was passed several times through a $26^{3/8}$ gauge needle (to shear the DNA) and mixed with 500 µl of phenol saturated with water, 100 µl of chloroform:isoamyl alcohol (49:1) and 50 µl of 2 M sodium acetate, pH 4.0. After centrifugation at 10,000g for 15 min at 4°C, the upper phase was collected and precipitated with one volume of isopropyl alcohol. Total RNA was pelleted by centrifugation at 10,000g for 5 min at 4°C, resuspended in 400 µl solution D and precipitated again with isopropyl alcohol. Total RNA was resuspended in 100 µl of 40 mM Tris-HCl. pH 7.9; 100 mM NaCl; 60 mM MgCl₂ and contaminating DNA was digested with 5 units of RQ1 DNAse (Promega) for 30 min at 37°C. Total RNA was extracted with phenol:chloroform, precipitated in ethanol and resuspended in 20 µl of

9.2 Northern analysis

RNA (1 to 20 µg) was denatured, electrophoresed through a 1.2% agaroseformaldehyde gel (9) and transferred to nylon membrane using the PosiBlot Pressure Blotter (Stratagene) as described by the manufacturer. RNA was cross linked to the nylon membrane by UV irradiation (1.2 x 10⁶ µJoules) using the StrataLinker from Stratagene. Prehybridization and hybridization of membranes was carried out at 42°C in 25 mM KPO₄, pH 7.4; 0.75 M NaCl; 75 mM sodium citrate, pH 7.0; 0.1% FicoIl 400; 0.1% polyvinylpyrrolidine; 0.1% bovine serum albumine; 10% dextran sulfate. A minimum of 1 x 10⁶ cpm of ³²P-labeled, random-primed cDNA probe (see below) was added per millilitter of hybridization solution. Blots were washed four times in 15 mM NaCl; 1.5 mM sodium citrate, pH 7.0; 0.1% SDS for 15 min. at room temperature and once in the same solution at 65°C for 20 min. Blots were rinsed in 15 mM NaCl; 1.5 mM sodium citrate, pH 7.0 and submitted to autoradiography. Restriction fragments were labeled with $[\alpha^{-32}P]$ dATP (Amersham) by the random priming method using the oligolabeling kit from Pharmacia. Unincorporated nucleotides and salts were removed on G-25 Sephadex column (Pharmacia). The neo cDNA probe was a 900bp Pst1 fragment derived from pMV-7. The IkBa cDNA probes was the 1.1 kbp EcoR1 fragment excised from pMV-7/IkBa plasmid. The IkBg cDNA probe was the 1.8 kb EcoR1 fragment derived from pMV-7/IkBg plasmid. The IkBg cDNA probe was the 1.8 kb EcoR1 fragment derived from pMV-7/IkBg plasmid. The HIV-1 proviral cDNA probes were the 2 and 2.2 kbp Hind-III fragments derived from pSVC21 BH10 and the β -actin cDNA probe was the 1 kbp Pst-1 fragment derived from p β -actin plasmid.

10 Analysis of HIV-1 multiplication in a single cycle infection model

Cos-1 cells were transfected with 10 µg of HIV-1 proviral DNA (pSVC21 BH10) and with 1, 5 or 10 µg of IkBa expressing plasmid: either SVK3/IkBa, SVK3/IkBa(2N), SVK3/IkBa(3C), or SVK3/IkBa(2N + 3C). In all experiments, the total amount of DNA transfected was completed to 20 µg with unrelated DNA (pUC8). DNA was introduced into cells by lipofection (LipofectamineTM) according to manufacturer's instructions (Promega inc.). Three days after transfection, the media and the cells were collected. The relative amount of virion protein p24 present in the media was determined by ELISA (233). Proteins were extracted from a portion of cells. Proteins were analyzed by immunoblotting as described above using IkBa monoclonal antibody, human sera from a HIV-1 seropositive individual or actin monoclonal antibody (ICN). Total RNA was extracted from the remaining cells using the RNeasy kit from Qiagen according to manufacturer's instructions. RNA was denatured, electrophoresed through a 1.2% agarose gel in formaldehyde buffer and was transferred to nylon membrane. Hybridization was carried out using ³²P labeled, random-primed HIV-1 proviral DNA or β -actin cDNA probes.

Chapter III

Disruption of IkBa Regulation by Antisense RNA Expression Leads to Malignant Transformation

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Several lines of evidence link the NF- κ B family of transcription factors to cell growth regulation and leukemogenesis. To further understand the association of I κ B with cell growth regulation, the effect of ectopic expression of sense and antisense I κ B genes was examined in NIH 3T3 cells.

Establishment of NIH 3T3 cells expressing $I_KB\alpha$ and $I_KB\gamma$. Human $I_KB\alpha$ and $I_KB\gamma$ cDNAs were subcloned into the retroviral expression vector pMV-7, downstream of the murine Moloney leukemia virus (MMoLV) 5' long terminal repeat (LTR) in sense and antisense orientations (Figure 11). These constructs, together with the pMV-7 retroviral construct as a control, were introduced into the amphotropic packaging cell line DAMP by electroporation and recombinant viruses were harvested from G418-resistant cells. NIH 3T3 cells were infected with recombinant viruses and selected in G418. Drug resistant colonies were isolated and characterized for IxB expression at the RNA and protein levels.

Clones infected with pMV-7/I κ B α (AS) retrovirus (I κ B α (AS) clones) were screened initially by Northern blot analysis using a neo cDNA probe. In up to 70% of the I κ B α (AS) clones a 4.4 kb transcript was detected (Figure 12A, lanes 2, 4, 6, 8-11). In a representative control clone infected with pMV-7 retrovirus (pMV-7 clone), a shorter transcript of 3.3 kb was detected (Figure 12A, lane 1). Using an I κ B α specific probe, high levels of the 4.4 kb I κ B α -containing retroviral mRNA were detected after an autoradiographic exposure of 30 min or 3h (Figure 12B, lanes 3, 4, and 7, 8 respectively), demonstrating the presence of I κ B α sequences in the retroviral transcript. A shorter 1.8 kb transcript representing the endogenous I κ B α mRNA was also detected in control pMV-7 clone 34 after the longer exposure (Figure 12B, lane 5 and 6). PMA was included in the experiment to determine if increased I κ B α mRNA (Figure 12B, lanes 5 and 6).



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Figure 11. Schematic representation of the pMV7/I κ B retroviral vectors. The location of important elements of the pMV-7/I κ B retroviral vectors are represented in the circular schematic of the plasmid including: the long terminal repeat of Moloney murine leukemia virus (MMLV LTR), the thymidine kinase promoter (TK promoter) the neomycin resistance gene (neo), the ampicilin resistance gene (amp) and the polyoma virus origin of replication (Py ori). Below the vector are the representative I κ B α and I κ B γ cDNA clones that were inserted into the EcoR1 site of pMV-7. The arrows below the genes indicate the sense and antisense orientations and the names of the representative clones.

Figure 12. Expression of $I\kappa B\alpha$ RNA and protein in $I\kappa B\alpha$ (AS) clones. A. Detection of $I\kappa B\alpha$ RNA using neo cDNA probe in 20 µg of total RNA isolated from G418 resistant clones infected with pMV-7/I $\kappa B\alpha$ (AS) (lanes 2 to 11) and control pMV-7 retroviruses (lane 1).

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Figure 12... B. $I\kappa B\alpha$ mRNA was detected by Northern blotting using an $I\kappa B\alpha$ cDNA probe and 3 µg of polyadenylated RNA isolated from control pMV-7 clone no. 34 (lanes 1, 2, 5, 6) and $I\kappa B\alpha(AS)$ clone no. 28 (lanes 3, 4, 7, 8). Cells were either untreated (lanes 1, 3, 5, 7) or stimulated by phorbol 12-myristate 13-acetate (PMA, 25 µg/ml) for 4 hours (lanes 2, 4, 6, 8). Lanes 1-4 represent a 30 min autoradiographic exposure while lanes 5-8 represent a 3h exposure of the same blot.

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..... **Figure 12...** C. I κ B α was detected in whole cell extracts (70 µg) from control pMV-7 clones no. 34 and no. 38 (lanes 1 and 2), parental NIH 3T3 cells (lane 5) and I κ B α (AS) clones no. 21 and no. 28 (lanes 3 and 4) by immunoblotting using a rabbit polyclonal antibody directed against human I κ B α (216).

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To examine whether $I\kappa B\alpha$ antisense RNA production affected the steady state levels of $I\kappa B\alpha$ protein, immunoblot analysis was performed on whole cell extracts from pMV-7 and $I\kappa B\alpha(AS)$ clones and compared to the parental NIH-3T3 cells. Immunoblot analysis demonstrated that endogenous $I\kappa B\alpha$ was present at 4 fold lower levels in $I\kappa B\alpha(AS)$ clones (Figure 12C, lanes 3 and 4) than in parental NIH 3T3 cells or in control pMV-7 clones (Figure 12C, lanes 1, 2 and 5). This result indicated that antisense $I\kappa B\alpha RNA$ interfered with the expression of endogenous $I\kappa B\alpha$ protein.

All G418 resistant colonies infected with the pMV-7/IsBa(S) retrovirus expressed a 3.5 kb mRNA (using a neo probe) deleted for the $I\kappa B\alpha$ sequence (data not shown), suggesting that the retroviral expression strategy allowed for selection of clones deleted for IxB α sequences, possibly because IxB α was deleterious to cell growth. Therefore, NIH 3T3 cells were co-transfected with plasmids pSV2neo and the $I\kappa B\alpha$ expression plasmid pSVK3/IxB α (120). G418 resistant colonies were isolated and IxB α overexpressing clones ($I_{\kappa}B\alpha(S)$ clones) were identified by immunoblot analysis. Of 21 clones screened for human $I\kappa B\alpha$ expression by immunoblot using an $I\kappa B\alpha$ anti-peptide antibody (AR20), 7 clones expressed human $I\kappa B\alpha$ at detectable but different levels (data not shown). The expression of IxB α protein in four IxB α (S) clones is illustrated in Figure 13, lanes 5-8; the I κ B α (S) clone no. 10 was the highest expressing clone, with 3 to 4 fold higher IxB α levels than other clones expressing the human protein (Figure 13, lane 7). The doublet of $I \times B \alpha$ (Figure 13, lanes 5 and 8) likely represents phosphorylated $I \times B \alpha$, as previously demonstrated (23). Four control clones (pSVK3 clones), generated by cotransfection with pSV2neo and pSVK3 and selection in G418, are also illustrated; no human IxBa was detected in these clones (Figure 13, lanes 1-4). On Figure 13, the short autoradiographic exposure does not allow detection of murine IkBa.

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IxB γ (S) and IxB γ (AS) clones expressing a 5.2 kb viral transcript were also isolated: ~ 70% of the IxB γ (S) clones expressed the retroviral transcript (data not shown). Synthesis of the 70 kDa IxB γ protein was detected in IxB γ (S) clones (Figure 14, lanes 4-6) but not Figure 13. Detection of $I \kappa B \alpha$ protein in $I \kappa B \alpha(S)$ clones. $I \kappa B \alpha$ was detected in whole cell extracts (10 µg) from control SVK3 clones no. 1, no. 2, no. 3 and no. 17 (lanes 1-4), and $I \kappa B \alpha(S)$ clones no. 2, no. 5, no. 10 and no. 14 (lanes 5-8) by immunoblotting using a rabbit polyclonal antibody directed against human $I \kappa B \alpha$ (216).

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Figure 14. Expression of $I\kappa B\gamma$ protein in $I\kappa B\gamma(S)$ clones. $I\kappa B\gamma$ was detected in whole cell extracts (10 µg) from control pMV-7 clones no. 34, no. 38 and no. 48 (lanes 1-3), and $I\kappa B\gamma(S)$ clones no. 8, no. 9 and no. 21 (lanes 4-6) by immunoblotting using a rabbit polyclonal antibody directed against the carboxyl-terminal end of NF κ B1/p105 (AR25) (216).

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In the control pMV-7 clones (Figure 14, lanes1-3) by immunoblot analysis using a Cterminal NF κ B1/p105 specific antibody (216). The endogenous murine NF κ B1/p105 protein was also detected in all lanes with this antibody (Figure 14). The construct used to express I κ B γ protein retained the proteolytic cleavage site of NF κ B1/p105; cleavage of a portion of I κ B γ likely explains the appearance of an I κ B γ doublet both by immunoblot analysis (Figure 14, lanes 4-6) and by immunoprecipitation (data not shown).

Growth characteristics of $I \ltimes B \alpha(AS)$ and $I \ltimes B \alpha(S)$ expressing clones Of the clones isolated from each set of infections, $I_{\kappa}B\alpha(S)$ and $I_{\kappa}B\alpha(AS)$ clones displayed newly acquired growth characteristics whereas $I_{K}B_{\gamma}(S)$ and $I_{K}B_{\gamma}(AS)$ clones maintained growth characteristics similar to control pMV-7 transduced cells (summarized in Tables 1 and 2). With $I \ltimes B \alpha(AS)$ clones, the first apparent change was the acquisition of a spindle shape cell morphology commonly observed in transformed fibroblasts (Figure 15B). This change was not observed in the control pMV-7 clones or with the $I\kappa B\gamma(S)$ and IkBy(AS) clones which displayed a flat morphology similar to NIH 3T3 cells (Figure 15A). Moreover, $I\kappa B\alpha(AS)$ clones lost density dependent control of cell growth as indicated by a three to fourfold increase in saturation density compared to parental NIH 3T3 cells (Table 1). The $I \times B \alpha(AS)$ clones also displayed anchorage-independent growth in soft agar at 10% efficiency (Figure 15D and Table 1). Most strikingly, subcutaneous injection of two I κ B α (AS) clones (no. 21 and no. 28) into three nude mice each resulted in tumor formation 16 to 24 days following injection. Malignant transformation was induced specifically by antisense IkBaRNA overexpression since clones overexpressing antisense or sense IkBy RNA possessed a biological phenotype indistinguishable from the parental NIH 3T3 cells (Table 1).

Complementing the observation that $I\kappa B\alpha(AS)$ clones induced cellular transformation, overexpression of $I\kappa B\alpha$ in $I\kappa B\alpha(S)$ clones decreased saturation density in media containing either 2% or 10% serum compared to transfected control pSVK3

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Figure 15. Growth characteristics of $I \kappa B \alpha(AS)$ clones. A. $I \kappa B \gamma(S)$ cells display a flat morphology similar to that of parental NIH 3T3 cells (not shown). B. $I \kappa B \alpha(AS)$ cells exhibit a spindle shape morphology. C. $I \kappa B \gamma(S)$ cells cultured three weeks in soft agar. D. $I \kappa B \alpha(AS)$ clones display anchorage independent growth. Magnification: 100x.

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Cell lines	Saturation density (2% serum)* (x10 ⁶)	Colony formation in soft agar *	Tumorigenicity +	
			Animals with tumors / animals injected	Latency (days)
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NIH 3T3	0.4 ± 0.0	0%	ND	NA
	n=1	n=1		
pMV-7	0.4 ± 0.1	0%	0/6	>60
	n=3	n=3	n=2	
ΙκΒα(AS)	1.4 ± 0.4	10 % ± 3	6/6	16-24
	n=4	n=3	n=2	
ΙκΒγ (S)	0.4 ± 0.1	0 %	0/6	>60
	n=3	n=3	n=2	
InBy (AS)	0.3 ± 0.1	0 %	ND	NA
•	n=3	n=2		

Table 1. Growth properties of retroviral transduced NIH 3T3 clones.

*: Numbers represent the mean value obtained for n clones analyzed \pm the standard deviation. †: Assay for tumorigenicity was performed using two independently isolated pMV-7, IxBa (AS), and IxBy(S) clones; three mice per clone were injected. The time required to produce tumors of at least 5 mm diameter was considered the latency period. ND: not determined. NA: not applicable. >60: mice did not show sign of tumor development 60 days after injection and were sacrificed.

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clones and did not promote anchorage independent growth in soft agar (Table 2), indicating that $I\kappa B\alpha$ overexpression inhibited cell growth. Also $I\kappa B\alpha(S)$ clones uniformly acquired a more flattened morphology compared to control cells transformed with the pSVK3 vector alone (data not shown). This newly acquired morphology may in part contribute to the reduced saturation density of $I\kappa B\alpha$ expressing cells. Injection of two $I\kappa B\alpha(S)$ expressing clones into nude mice did not cause tumors (0/6).

NF-κB binding activity in IκBα(AS) and IκBα(S) expressing cells. To determine if reduced IκBα expression (Figure 12C) affected NF-κB activity, the HIV enhancer probe (-105 to -80) which contains two NF-κB sites was used in electrophoretic mobility shift assays (EMSA) to assess NF-κB DNA binding activity in nuclear extracts from control pMV-7 and IκBα(AS) clones (Figure 16). NF-κB complex formation was fivefold more efficient with extracts from IκBα(AS) clones than from control pMV-7 clones (Figure 16, lanes 1, 4, 7, 10). Specificity of NF-κB complex formation was demonstrated by bandshift competition with an excess of wild type HIV enhancer DNA (Figure 16, lanes 3, 6, 9, 12) but not with an oligonucleotide mutated in the NF-κB binding sites (Figure 16, lanes 2, 5, 8, 11). This biochemical phenotype was not stable and with time in culture constitutive NF-κB activity was reduced, without an effect on the growth phenotype. No quantitative differences in complex formation could be detected in IκBγ(S), IκBγ(AS) or IκBα(S) cells (data not shown).

Shifted shift experiments were also performed using subunit specific antisera (NF κ B1/p50, RelA(p65), NF κ B2/p52, c Rel) to determine which NF- κ B proteins were present in complexes in I κ B α (AS) and I κ B α (G) expressing cells (Figure 17). Specificity of the shift complex was confirmed by competition using an excess of peptide against which the antibody was raised (216). Based on this assay, RelA(p65) was the main NF- κ B subunit identified in the protein-DNA complex using unstimulated cell extracts (Figure 17, lane 4). After induction with TNF- α , qualitative and quantitative changes in

Cell line	Saturation density (2% serum) (x10 ⁶)	Saturation density (10% serum) (x10 ⁶)	Doubling time in 10% serum (hours)	Colony formation in soft agar
NIH 3T3	0.4 ± 0.0	1.1 ± 0.1	21.9 ± 3.0	0%
	n=1	n=1	n=1	n=1
SVK 3	0.7 ± 0.4	1.4 ± 0.2	19.6 ± 1.1	$0.6\% \pm 0.4$
	n=4	n=3	n=3	n=4
InBa (S)	0.3 ± 0.1	0.6 ± 0.2	20.2 ± 1.1	0%
	n=4	n=3	n=3	n=4

Table 2. Growth properties of transfected NIH 3T3 clones.Numbersrepresent the mean value obtained for n clones analyzed \pm the standard deviation.

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Figure 16. NF- κ B binding activity in I κ B α (AS) clones. Mobility shift analysis of proteins binding to the HIV enhancer probe was performed with nuclear extracts (5 µg) from pMV-7 control clones no. 34 and no. 38 (lanes 1-6) and I κ B α (AS) clones no. 21 and no. 28 (lanes 7-12). The NF- κ B specific complex is indicated by the arrow; lanes marked Mut or Wt contained a 200 fold excess of either a mutated HIV enhancer oligonucleotide (Mut) or wild type HIV enhancer oligonucleotide (Wt), as described previously (236).



Figure 17. Subunit composition of NF-xB complexes.

Nuclear extracts (2 µg) from untreated (lanes 1-7) and TNF- α treated (4h) (lanes 8 to 14) I κ B α (AS) cells were analysed by EMSA in presense of antisera specific to NF κ B1/50 (lane 2, 3, 9, 10), RelA(p65) (lanes 4, 5, 11, 12) or NF κ B2//52 (lanes 6, 7, 13, 14). Competing peptide (2 µg) was added to the reaction to demonstrate specificity of the protein-antibody interactions

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the composition of subunits forming DNA binding complexes were observed. In addition to high levels of RelA(p65) subunit binding (Figure 17, lane 11), NFxB1/p50 and NFxB2/p52 subunits were also detected in NF-xB-DNA complexes (Figure 17A, lanes 9 and 13); no c-Rel binding activity was detected in uninduced or induced extracts (data not shown). These observations indicate that decreasing the endogenous IxBa protein level resulted in higher levels of nuclear NF-xB complex in IxBa(AS) cells, composed mainly of RelA(p65). TNF- α induction resulted in increased levels of NF-xB binding activity composed of RelA(p65), as well as NFxB1/p50 and NFxB2/p52. Similarly, in clones expressing IxBa(S) and IxBy(S). TNF- α treatment induced NF-xB complexes that contained RelA(p65), NFxB1/p50 and NFxB2/p52 (data not shown).

NF- κ B dependent gene expression in $I\kappa B\alpha(S)$ and $I\kappa B\alpha(AS)$ expressing cells. To examine the consequences of increased NF-xB specific DNA binding activity. expression of NF- κ B regulated CAT reporter constructs was examined in I κ B α (S) and IkBa(AS) clones (Figure 18). Constructs containing a single copy of the HIV-1 enhancer (-105 to -80) which contains two tandem NF-kB binding sites, or a mutated copy of the enhancer (161) linked to the basal SV40 promoter (pHIV-CAT and pHIV(mut)-CAT, respectively) were introduced into the pMV-7 or $I\kappa B\alpha(AS)$ and the SVK3 or $I\kappa B\alpha(S)$ clones by liposome mediated transfection. Relative CAT activity for pHIV(mut)-CAT and pHIV-CAT were quantified and expressed as a ratio relative to the level of pSV1 CAT activity in each cell line. Relative levels of CAT activity were increased about threefold (average of three experiments) in $I\kappa B\alpha(AS)$ clones compared to pMV-7 control clones, indicating that increased NF-kB binding activity correlated with an increased reporter gene expression. Relative NF-xB reporter gene activity was also examined in cells overexpressing IxB α and in their respective SVK3 control cells. IxB α (S) cells displayed a reduced CAT activity when transfected with pHIV-CAT plasmid compared to control SVK3 cells or $I \times B\alpha(AS)$ clones (Figure 18), indicating that increased $I \times B\alpha$

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Figure 18. Activity of NF- κ B dependent reporter constructs in I κ B α (AS) and I κ B α (S) clones. Control pMV-7, I κ B α (AS), control SVK3 and I κ B α (S) clones were transfected with the pSV1 CAT, pHIV(mut)-CAT or pHIV-CAT reporter plasmids (10 mg). At 48h after transfection, extracts were prepared and analyzed for cumulative CAT activity (100 µg for 3h). Relative CAT activity for pHIV(mut)-CAT (hatched bar) and pHIV-CAT (solid bar) were expressed as a ratio relative to the level of pSV1 CAT activity in each cell line.

expression correlated with a decreased reporter gene expression. $I\kappa B\gamma(S)$ and $I\kappa B\gamma(AS)$ cells were also tested, but no significant difference in reporter gene expression was observed (data not shown).

Expression of p50/65 chimeric protein in NIH 3T3 cells. Overexpression of antisense I κ B α RNA induced malignant transformation and increased NF- κ B activity in the cell. Previous experiments demonstrated that a fusion protein between the Rel homology domain of NF κ B1 and the C-terminal domain of ReIA(p65) generated a chimeric protein with strong trans-activation potential *in vitro* (166). We thus predicted that overexpression of p50/65 may increase NF- κ B activity *in vivo* and lead to malignant transformation. To test the *in vivo* activity of p50/65, 3T3 clones were also generated that expressed the p50/65 fusion protein. Immunoblot analysis of 3T3 clones using the anti-ReIA(p65) AR28 antisera clearly demonstrated that the 75kDa p50/65 protein was expressed at levels equivalent to murine ReIA(p65) (Figure 19, lanes 3-4).

NF- κ B binding activity in p50/65 expressing cells. The molecular weight of the p50/65 fusion protein (75 kDa) resulted in the formation of a distinct, slowly migrating protein-DNA complex that was unique to p50/65 expressing cells (Figure 20A, lanes 5-8). Some p50/65 binding activity was constitutively present in p50/65 expressing cells, particularly in clone no. 13 (Figure 20A, lanes 5 and 6) and DNA binding activity was highly inducible by TNF- α induction (Figure 20A, lanes 7-8).

To confirm that the novel protein-DNA complex observed by EMSA was indeed composed of p50/65. supershift analyses using antibodies to NFxB1/p50, RelA(p65) and NFxB2/p52 were performed (Figure 20B); no detectable c-Rel activity was observed in these cells (data not shown). When the N-terminal, human specific anti-NFxB1/p50 antibody was used with extracts from uninduced or TNF- α induced cells (Figure 20B, lanes 1-6), the entire NF-xB complex was shifted (Figure 20B, lanes 2 and 5); likewise,

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Figure 19. Expression of p50/65 chimeric protein in NIH 3T3 cells. p50/65 fusion protein as well as murine RelA(p65) was detected in whole cell extracts (25 μ g) from control SVK3 clones no. 3 and no. 17 (lanes 1 and 2), and p50/65 clones no.7 and no.13 (lanes 3 and 4) using AR28 anti-RelA(p65) antiserum.



Figure 20. NF- κ B binding activity in p50/65 expressing cells. (A) Nuclear extracts (5 µg) from unstimulated (lanes 1, 2, 5, 6) and TNF- α stimulated (lanes 3, 4, 7, 8) cells were analyzed for NF- κ B binding activity by EMSA using the ³²P-labeled HIV-1 enhancer probe. Clones used were control SVK3 clones no. 3 and no. 17 (lanes 1-4), and p50/65 clones no. 7 and no. 13 (lanes 5-8). The p50/65 specific complex is indicated by an arrow and the NF- κ B complex is indicated by the bracket.



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Figure 20... (B) Identification of NF- κ B binding subunits. Nuclear extracts (5 µg) used in (A) were subjected to a supershift EMSA; extracts were preincubated with antisera AR27 against NF κ B1/p50 (lanes 1-6), AR28 against RelA(p65) (lanes 7-10), and AR43 against NF κ B2/p52 (lanes 11-14) and analyzed by EMSA. Competing peptide (2 µg) was used to demonstrate the specificity of the supershift (lanes marked +).



Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14

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Figure 20... (C) Co-immunoprecipitation of murine RelA(p65), $1 \times B \alpha$ and human p50/65. Control SVK3 clone no. 17 and p50/65 clone no. 13 were lysed in TNT buffer, cell lysates incubated with anti-I $\times B \alpha$ antibody and protein A-sepharose, and $1 \times B \alpha$ eluted with AR20 peptide. Immunoblotting for RelA(p65) and p50/65 was performed using antiserum AR28.

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the anti-RelA(p65) antibody shifted both the p50/65 and the NF-xB complex (Figure 20B, lanes 7 and 9). Thus, anti-NFxB1/p50 and anti-RelA(p65) antisera confirmed that the slowly migrating complex was composed of a chimeric protein recognized by the N-terminal specific NFxB1/p50 antibody and the C-terminal specific RelA(p65) antibody. Also, murine NFxB2/p52 participated in complex formation in p50/65 expressing cells (Figure 20B, lanes 11 and 13) but did not associate with p50/65, since a loss of the p50/65 complex was not observed when anti-NFxB2/p52 antibody was used (Figure 20B, lanes 11 and 13). TNF- α induction increased overall DNA binding activity, yet the relative composition of the complexes remained unchanged (compare Figure 20B, lanes 1 and 4).

To determine if the p50/65 fusion protein interacted with $I\kappa B\alpha$ as efficiently as RelA(p65), immunoprecipitation analysis was performed using $I\kappa B\alpha$ antibody (AR20). p50/65 was detected in the immunoprecipitate by subsequent blotting with anti-RelA(p65) antibody (Figure 20C). In SVK3 cells, p65 associated with $I\kappa B\alpha$ (Figure 20C, lane 2), and in p50/65 expressing cells the chimeric p50/65 protein, as well as murine RelA(p65), associated with $I\kappa B\alpha$ (Figure 20C, lane 4). Compared to the total amounts of p50/65 and RelA(p65) in the cells (Figure 19), about 4-fold less p50/65 fusion protein interacted with $I\kappa B\alpha$ compared to RelA(p65), indicating that the p50/65 fusion protein was recognized less efficiently by $I\kappa B\alpha$ than RelA(p65). Relatively weak p50/65 I $\kappa B\alpha$ interactions may contribute to the higher basal and constitutive levels of p50/65 binding activity. The lower band in all lanes was non-specific and did not compete with peptide (Figure 20C, lanes 1 and 3).

Growth characteristics of p50/65 expressing clones As opposed to $I\kappa B\alpha(AS)$ clones, p50/65 clones displayed a flat morphology similar to NIH 3T3 cells and they retained density dependent control of cell growth as indicated by the absence of change in saturation density compared to control SVK3 clones (Table 3). Moreover, p50/65 clones

Cell lines	Saturation density (2% serum)* (x10 ⁶)	Colony formation in soft agar *	Tumorigenicity †	
			Animals with tumors / animals injected	Latency (days)
SVK3	0.7 ± 0.4 n=4	0.6 ± 0.4 % n=4	0/6 n=2	>45
p50/65(S)	0.3 ± 0.2 n=3	0.6 ± 0.6 % n=3	0/3 n=1	>45

Table 3. Growth properties of p50/65 transfected NIH 3T3 clones.

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*: Numbers represent the mean value obtained for n clones analyzed \pm the standard deviation. \dagger : Assay for tumorigenicity was performed using n independently isolated clones; three mice per clone were injected. The time required to produce tumors of at least 5 mm diameter was considered the latency period. >45: mice did not show sign of tumor development 45 days after injection and were sacrificed.

did not display anchorage-independent growth in soft agar and subcutaneous injection of p50/65 clone no. 17 into three nude mice did not result in tumor formation up to 45 days following injection (Table 3).

To examine if overexpression of p50/65 modulated the transcription of NF-xB dependent genes, analysis of NF-xB regulated IxB α RNA levels was performed using human IxB α cDNA as a probe. Surprisingly, IxB α mRNA levels did not vary significantly in p50/65 expressing cells compared to SVK3 control cells (data not shown). Furthermore, the NF-xB regulated CAT reporter constructs pHIV-CAT was introduced into SVK3 and P50/65 clones by liposome mediated transfection but no significant difference in CAT activity was measured between the two cell types (data not shown). Therefore, it appears that p50/65 overexpression did not mimic anti-sense IxB α overexpression since it was incapable to upregulate NF-xB activity and NIH-3T3 cells were not transformed by p50/65.

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Chapter IV

Phosphorylation of $I \ltimes B \alpha$ in the C-terminal PEST Domain by Casein Kinase II affects intrinsic protein stability

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The previous chapter demonstrates that reduced IxB α levels induced by anti-sense RNA overexpression leads to malignant transformation in rodent fibroblasts. The control of IxB α stability appears therefore to be a key factor in cell growth control. IxB α degradation upon cell stimulation is preceded by and induced by IxB α hyperphosphorylation. The aim of the study described in the present chapter was to identify the kinase which triggers IxB α degradation. This study revealed that casein kinase II constitutively phosphorylates IxB α and contributes to the inherent instability of the protein. The isolation and the characterization of the IxB α associated kinase was performed by Dr. Rongtuan Lin and is summerized below.

Detection of a protein kinase activity binding to I \times B \alpha. A protein kinase activity isolated from Jurkat T cells that specifically interacted with and phosphorylated recombinant IxBa, was characterized in our laboratory. IxBa was expressed as a polyhistidine-tagged protein in E. coli, was bound to His-Bind metal chelation resin and incubated with whole cell extract derived from Jurkat T cells. After washing the column, the kinase activity was eluted with 6M urea and assessed using an in gel kinase assay with GST-IkBa as substrate. Two kinase activities migrating at 43 kDa and 38 kDa were detected. The kinase activities were not affected by inducers of NF-κB DNA binding activity such as TNF- α , anti-CD3 antibody, poly rI:rC, or PMA, suggesting a constitutive kinase activity. This protein kinase was identified as CKII based on the following biochemical and immunological criteria: 1) The kinase preferred Mn++ and Mg⁺⁺ as divalent cations, was stimulated by Na⁺ and K⁺, was inhibited by heparin, and utilized ATP or GTP as a phosphate donor. All of these characteristics are consistent with that of CKII. 2). The bound proteins eluted from the IkBa affinity column contained the CKII catalytic subunit as detected immunoblotting using CKIIa antibody. and immunodepletion of the extract with CKIIa antibody eliminated the IkBa specific kinase activity.

Mapping the casein kinase II phosphorylation sites in I κ B α . To identify the region of I κ B α specifically phosphorylated by the I κ B α -associated kinase, a series of C-terminal deletions were constructed that generated I κ B α proteins of 260 aa (Δ 1), 268 aa (Δ 2), 287 aa (Δ 3) and 295 aa (Δ 4) (Figure 21). The truncated I κ B α proteins were used as substrate for *in vitro* protein phosphorylation assays with the kinase partially purified by affinity chromatography. Results of these experiments suggested that the phosphorylation sites were located in the C-terminal PEST-like sequence (Figure 21). In I κ B α (Δ 1) and I κ B α (Δ 2), no phosphorylation was detected. The I κ B α (Δ 4) was phosphorylated as efficiently as wild type I κ B α protein, whereas the I κ B α (Δ 3) was phosphorylated, but with lower efficiency. Analysis of these deletions suggested that more than one phosphorylation site was present in the PEST-like sequence of I κ B α , with at least one site located between residues 269 and 287 and another between residues 288 and 295.

Serine and threonine residues within the region spanning as 269 to 295 were specifically mutated to alanine in order to identify critical phosphoamino acid residues. Mutations in Ser-283 (S283A). Ser-288 (S288A), or Ser-293 (S293A) had little or no effect on the *in vitro* phosphorylation of IkB α by its associated kinase (Figure 21), while mutation in Thr-291 (T291A) reduced the phosphorylation by 2- to 4-fold (Figure 21). However, the double mutation S283A, T291A (mutant IkB α (DM)) or the S283A, T291A, T299A triple mutation (mutant IkB α (3C)) reduced phosphorylation up to 20 fold (Figure 21). Importantly, each of Ser-283, Thr-291, and Thr-299 lies within a consensus casein kinase II phosphorylation site (S/T-X-X-E/D).

To further characterize the effect of point mutations on the phosphorylation state of $I\kappa B\alpha$, the target amino acid residues on wtI $\kappa B\alpha$, $I\kappa B\alpha(T291A)$, and $I\kappa B\alpha(3C)$ were determined by phosphoamino acid analysis (61). Phosphoamino acid analysis of $I\kappa B\alpha$ proteins (equal amounts of cpm) demonstrated that both serine and threonine residues were phosphorylated by the kinase. Strikingly, the ratio of phosphothreonine: phosphoserine was 4:1 with wtI $\kappa B\alpha$, while this ratio was inversed



Figure 21. In vitro phosphorylation of $I \kappa B \alpha$ by the $I \kappa B \alpha$ -associated kinase. The 317 amino acids of the human $I \kappa B \alpha$ protein is illustrated schematically. The hatched boxes represent the ankyrin repeat domain; the open box represents the N-terminal domain (aa 1-73) and the shaded box represents the C-terminal PEST-like domain (aa 281-317). The amino acid sequence of the C-terminal region is shown from residue 261 to residue 317. The end-point of the deletions are indicated by the solid line. For point mutations, the substituted alanine residues are indicated. Point mutations are described on the left: in one-letter code as wild-type amino acid, the residue number, and the mutant amino acid. Phosphorylation *in vitro* by the I κ B α specific kinase is summarized on the right. (1:4) with the I κ B α (T291A) mutant. Similarly, with I κ B α (3C), the pT:pS ratio was reversed (1:2) compared to wtI κ B α .

Detection of phosphorylated I κ B α in vivo. In vivo, I κ B α is constitutively phosphorylated in addition to its inducible phosphorylation (43,73). To investigate whether the C-terminal CKII sites were required for I κ B α constitutive phosphorylation, stably transfected cells expressing wtI κ Ba, I κ Ba(3C) or control 3T3 tTA cells were labeled in vivo with ³²P-orthophosphate or ³⁵S-methionine. The IxBa proteins were purified by double immunoprecipitation, separated by SDS PAGE and detected by autoradiography (Figure 22). Endogenous murine $I_{\kappa}B\alpha$ was detected as a constitutively phosphorylated protein in all samples (Figure 22A, lanes 1-6), human wtI κ B α was detected in lanes 3 and 4, while the phosphorylation of $I\kappa B\alpha(3C)$ was undetectable (Figure 22A, lanes 5 and 6). Treatment with TNF- α in the presence of calpain inhibitor for 30 min also failed to reveal any phosphorylation of the IkB α (3C). TNF- α mediated phosphorylation of human wtIxB α was detectable with long exposure of the autoradiograph (data not shown). The failure to detect phosphorylation in $I\kappa B\alpha(3C)$ expressing cells was not due to the absence of protein, since expression of $I\kappa B\alpha(3C)$ was detectable by immunoblotting from the same membrane used in Figure 22A with a monoclonal human IxB α antibody MAD 10B (134) (Figure 22B lanes 5 and 6). Furthermore, the expression of $I\kappa B\alpha(3C)$ was also detectable when proteins were labeled with ³⁵S-methionine and purified by double immunoprecipitation (Figure 22C, lanes 5 and 6).

NIH 3T3 cells expressing wtI κ B α , or I κ B α (DM) under the control of the Tet responsive promoter were used to determine if the C-terminal mutations of I κ B α altered phosphorylation and/or degradation of I κ B α *in vivo*. Cells were stimulated with TNF- α (5 ng/ml) for 1, 5, or 60 min. (Figure 23); hyperphosphorylation of wtI κ B α was detected by the appearance of a more slowly migrating I κ B α band within 1 or 5 min. of TNF- α Figure 22. Constitutive phosphorylation in $I \ltimes B \alpha$ expressing NIH 3T3 cells. A and C. Control tTA-3T3 cells (lanes 1 and 2) or stably transfected wild type $I \ltimes B \alpha$ (lanes 3 and 4) and $I \ltimes B \alpha (3C)$ (lanes 5 and 6) expressing cells were labeled with ³²P-orthophosphate (A) or ³⁵S-methionine (C), untreated (lanes 1, 3 and 5) or treated with calpain inhibitor I (100 µM) and TNF- α (5 ng/ml) (lanes 2, 4 and 6). Extracts (300 µg) were then double immunoprecipitated with $I \ltimes B \alpha$ polyclonal antibody (AR20) and the immunoprecipitates were subjected to SDS PAGE, transfered to membrane and autoradiographed. B. The expression of human $I \ltimes B \alpha$ in ³²P-orthophosphate labeled cells was detected by western blot (WB) from the same membrane as used in panel A with MAD 10B monoclonal human $I \ltimes B \alpha$ (134).



Figure 23. TNF- α induced phosphorylation in IxB α expressing NIH 3T3 cells. Protein extracts (50 µg) from wtIxB α and IxB α (S283A,T291A) [termed IxB α (DM)] expressing cells were electrophoresed on a 7%-13% gradient SDS polyacrylamide gel, transferred to nitrocellulose and analyzed using IxB α antibody AR20. Hyperphosphorylated IxB α (IxB α -P) appears as a distinct slower migrating protein band. wtIxB α cells (lanes 1-5) and IxB α (DM) cells (lanes 6-10) were either left untreated (lanes 1, 6) or stimulated with 5 ng/ml TNF- α for 10 seconds (lanes 2, 7), 1 minute (lanes 3, 8), 5 min. (lanes 4, 9) or 60 min. (lanes 5, 10).



treatment (Figure 23, lanes 3 and 4), as described previously (23,284). The slowly migrating form of IkB α disappeared when extracts were treated with CIP but not when phosphatase inhibitors were included in the CIP reaction (data not shown), thus confirming the phosphorylated nature of IkB α . No phosphorylated IkB α was detected at 60 min after TNF- α treatment (Figure 23, lane 5), reflecting the rapid and transient nature of IkB α phosphorylation (23,284). IkB α (DM) - and in independent experiments IkB α (3C) - was only weakly phosphorylated 5 min. after TNF- α treatment as compared to wtIkB α (Figure 23, lane 9); however, both wtIkB α and IkB α (S283A,T291A) degraded to approximately the same extent by 60 min. after TNF- α treatment (Figure 23, lanes 5 and 10). These experiments suggest that mutations in the C-terminal CKII sites affect the overall level of IkB α phosphorylation but do not block IkB α degradation *in vivo*, thus complementing the results of Brown *et al.* (43) demonstrating the requirement for phosphorylation at Ser-32 and Ser-36.

Mutated C-terminal CKII sites increase $I_{\kappa}B\alpha$ intrinsic stability. To examine the effect of point mutations on the degradation of $I_{\kappa}B\alpha$, the turnover rate of $I_{\kappa}B\alpha$ was measured in cells stably expressing $I_{\kappa}B\alpha$ and treated with TNF- α in the presence of the protein synthesis inhibitor cycloheximide at different times after TNF- α addition (Figure 24). Following TNF- α stimulation, both wtI κ B α and $I_{\kappa}B\alpha(3C)$ degraded rapidly (Figure 24A, lanes 7-12). In contrast, independent experiments confirmed that $I_{\kappa}B\alpha(3GA)$ was resistant to TNF- α induced degradation (data not shown). Therefore the triple point mutation in $I_{\kappa}B\alpha-3C$ did not alter inducer mediated degradation. However, in the presence of cycloheximide but without inducer, $I_{\kappa}B\alpha(3C)$ appeared more stable than wild type protein (Figure 24A, lanes 1-6). To confirm that the mutation in the C-terminal sites affected the intrinsic stability of $I_{\kappa}B\alpha$ protein, NIH 3T3 cells were also transiently transfected with wtI κ B α or $I_{\kappa}B\alpha(3C)$, and $I_{\kappa}B\alpha$ turnover in the presence of cycloheximide A and $I_{\kappa}B\alpha(3C)$ and $I_{\kappa}B\alpha(3C)$ was

Figure 24. Analysis of $I \ltimes B \alpha$ turnover rate. A. NIH 3T3 cells stably transfected with tTA-I $\ltimes B \alpha$ (WT) and tTA-I $\ltimes B \alpha$ (3C) were treated with cycloheximide (Cxd) (50µg/ml) alone (lanes 1-6) or stimulated with TNF- α (5 ng/ml) in the presence of cycloheximide (lanes 7-12) for 0 (lanes 1 and 7), 15 (lanes 2 and 8), 30 (lanes 3 and 9), 60 (lanes 4 and 10), 90 (lanes 5 and 11) and 120 min. (lanes 6 and 12). I $\ltimes B \alpha$ was detected in whole cell extracts (15 µg) by immunoblotting with AR20 antibody.



Figure 24... B. NIH 3T3 cells transiently transfected with $I\kappa B\alpha(WT)$ and $I\kappa B\alpha(3C)$ (in pSVK3 vector) were treated with cycloheximide (50 µg/ml) for 0 (lane 1), 15 (lane 2), 30 (lane 3), 60 (lane 4), 90 (lane 5), 120 (lane 6), 180 (lane 7) and 240 min. (lane 8). I $\kappa B\alpha$ was detected in whole cell extracts (15 µg) by immunoblotting with AR20 antibody.

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about 90 min. compared to 30 min. for wtI κ B α , demonstrating that mutation of CKII phosphorylation sites increased I κ B α intrinsic stability but did not affect inducer mediated degradation.

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Chapter V

The Role of the C-terminal Domain of IκBα in Protein Degradation and Stabilization

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The previous chapter demonstrated that casein kinase II constitutively phosphorylates the C-terminus of $I\kappa B\alpha$ and thereby controls the intrinsic stability of $I\kappa B\alpha$. The CKII sites are located within a region rich in proline, glutamic acid, serine and threonine amino acids named the PEST domain (112,232) which is found in a number of short lived proteins. The study described in the present chapter examines the role of the Cterminal domain of $I\kappa B\alpha$ in regulating $I\kappa B\alpha$ function. Results demonstrates that although the PEST domain appears dispensable for $I\kappa B\alpha$ function, the adjacent domain protects $I\kappa B\alpha$ from the constitutive activity of the proteasome.

Inhibition of NF-\kappaB-DNA complex formation. To investigate the role of the I κ B α Cterminus in NF-xB/IxBa regulation, we generated a series of C-terminal deletions of I κ B α (Figure 25). The I κ B α proteins were generated by inserting an artificial stop codon in the human IkB α gene at as 261 (Δ 1), as 269 (Δ 2), as 288 (Δ 3) and as 296 (Δ 4), respectively. IxB α (DM) represents full length human IxB α in which the S283 and T291 were substituted for alanine residues and $I_{\kappa}B\alpha(3C)$ contains the S283A, T291A, and T299A substitutions (Figure 25). Wild type and C-terminal deletions of $I \kappa B \alpha$ were examined for their ability to inhibit NF-kB-DNA complex formation in an electromobility shift assay (EMSA). Extracts from tTA-3T3 cells stimulated with TNF- α for 30 min were analyzed for NF-xB binding activity using a ³²P-labeled probe corresponding to the PRDII region of the interferon β promoter (Figure 26). Addition of recombinant wtIxBa, IxBa($\Delta 4$), IxBa($\Delta 3$), IxBa(DM) and IxBa(3C) proteins reduced the intensity of the NF-xB/PRDII band more than 10-fold (Figure 26, lanes 2, 3, 4, 7 and 8), whereas addition of $I \kappa B \alpha(\Delta 1)$, or $I \kappa B \alpha(\Delta 2)$ had no affect on NF- $\kappa B \cdot DNA$ complex formation (Figure 26, lanes 5 and 6). This result demonstrated that the region located between aa 269 to aa 287 is important for inhibiting NF-kB-DNA complex formation in vitro.



Figure 25. Schematic representation of human IxB α and C-terminal deletion mutants. Human IxB α contains five internal ankyrin repeats (SWI6/ANK) involved in the binding to NF-xB molecules. At the N-terminal of IxB α there are two phosphorylation sites (S32 and S36), shown previously to play a role in inducer mediated degradation (42, 43). A region rich in proline, serine, threonine and glutamic acid, the PEST domain, spans aa 264 to 317. The N-terminal region of IxB α between aa 25 and 45, and the C-terminal region of IxB α between aa 251 and 317 are expanded above and below the schematic to show the one letter amino acid sequence. The Cterminal ends of the deletions IxB α (Δ 1)(aa1 to 260), IxB α (Δ 2) (aa 1 to 268), IxB α (Δ 3) (aa 1 to 287) and IxB α (Δ 4) (aa 1 to 295) are depicted. In mutant IxB α (DM), S283 and T291 were substituted for alanines and in IxB α (3C), T299 was also substituted for alanine. The C-terminal region involved in degradation (aa 279 to 287) is indicated in bold letters. The boundary aa 279 was determined in reference no. 300; the boundary aa 287 was determined in this study.

Figure 26. Dissociation of NF- κ B·DNA complexes by recombinant I κ B α . Nuclear extracts from tTA-3T3 cells (5 µg) stimulated for 30 min with TNF- α were incubated with ³²P-labeled probe (0.2 ng) corresponding to the interferon- β PRDII region (237). The NF- κ B·DNA complex was visualized on native 5% polyacrylamide gel (lane 1). The specificity of the complex formation was tested by adding a 200-fold molar excess of unlabeled wild type or mutated PRDII dsDNA to the reaction, prior to labeled probe addition (data not shown, see Materials and Methods). Recombinant wtI κ B α (lanes 2 and 9). I κ B α (Δ 4) (lanes 3 and 10). I κ B α (Δ 3) (lanes 4 and 11), I κ B α (Δ 2) (lanes 5 and 12). I κ B α (Δ 1) (lanes 6 and 13), I κ B α (DM) (lanes 7 and 14) or I κ B α (3C) (lanes 8 and 15) were added to the extracts prior to probe addition. The recombinant I κ B α proteins were either untreated (lanes 2-8) or phosphorylated *in vitro* with recombinant casein kinase II prior to addition to the EMSA reactions (lanes 9-15).



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In vivo, $I\kappa B\alpha$ is constitutively phosphorylated at the C-terminus by casein kinase II (17,165). Several previous reports demonstrated that the phosphorylation level of $I\kappa B\alpha$ influences the ability of $I\kappa B\alpha$ to dissociate NF- κB -DNA complexes (101,143,169). However, *in vitro* phosphorylation of wild type or mutant $I\kappa B\alpha$ proteins with casein kinase II (Figure 26, lanes 9 to 15) did not modulate the capacity of $I\kappa B\alpha$ to inhibit NF- κB /PRDII DNA complex formation *in vitro*.

Association of $I \ltimes B\alpha$ with RelA *in vitro*. A well characterized function of $I \ltimes B\alpha$ is the capacity to bind and retain NF- κB proteins in a latent form in the cytoplasm. To determine if $I \ltimes B\alpha$ mutants were still able to associate with NF- κB *in vitro*, GST-Np65 affinity columns were prepared and loaded with equivalent amounts of recombinant $I \ltimes B\alpha$ protein (10 ng). After extensive washing with HNTG buffer, the bound protein was eluted with reduced glutathione and analyzed by immunoblotting. All $I \ltimes B\alpha$ proteins tested were detected in the eluate from GST-Np65 columns but not GST columns (Figure 27, lanes 3, 6, 9, 12, 15, 18 and Table 4). With the exception of $I \ltimes B\alpha (\Delta 3)$ did not bind to GST alone (data not shown). Based on these results, deletion of the C-terminal region of $I \ltimes B\alpha$ between aa 261 to 317 did not alter the binding of $I \ltimes B\alpha$ to the Rel domain of RelA *in vitro*. These results are consistent with previous studies indicating that interaction between $I \ltimes B\alpha$ and NF- κB RelA was dependent upon the ankyrin repeat domain (80).

Inhibition of NF-\kappaB dependent gene expression. Next, the effect of $I\kappa B\alpha$ C-terminal truncations on the inhibition of NF- κ B dependent gene expression was examined. NF- κ B activity was detected by measuring CAT activity derived from pHIV-CAT reporter plasmid in N-Tera-2 cells which are deficient in NF- κ B activity (38,310). The level of NF- κ B was increased in these cells by co-expressing RelA and treating with PMA (43).
Figure 27. In vitro association of $I \ltimes B \alpha$ mutants with N-terminal RelA. Bacterial recombinant $I \ltimes B \alpha(\Delta 1)$ (lanes 10-12). $I \ltimes B \alpha(\Delta 2)$ (lanes 7-9), $I \ltimes B \alpha(\Delta 3)$ (lanes 4-6), $I \ltimes B \alpha(\Delta 4)$ (lanes 1-3), $I \ltimes B \alpha(DM)$ (lanes 13-15) and wt I \ltimes B (lanes 16-18) proteins (10 ng each) were incubated with glutathione agarose beads loaded with GST-Np65 fusion protein (lanes 3, 6, 9, 12, 15 and 18) or GST alone (lanes 2, 5, 8, 11, 14 and 17). The beads were washed three times in HNTG buffer. GST-Np65 or GST bound I $\ltimes B \alpha$ was eluted with 50 mM Tris-Cl, pH 8.0; 15 mM glutathione. One third of the eluate was boiled in SDS sample buffer and analyzed by immunoblotting using affinity purified I $\ltimes B \alpha$ antibody (AR20). Control lanes 1, 4, 7, 10, 13 and 16 show bands corresponding to approximately 2 ng of recombinant I $\ltimes B \alpha$ protein.



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ΙκΒα	Intrinsic stability (half life) ^a	TNF-α induced degradation (half life) ^b	LPS induced degradation (half life) ^C	Association with RelA in vitrod	Association with RelA <i>in</i> vivo ^e	Inhibition of NF-κB dependent transcription ^f	Dissociation of NF-xB·DNA complex <i>9</i>
wild type (wt)	~ 120 min	~5 min	~5 min	+	+	+	+
S283A; T291A (DM)	~ 120 min	~5 min	~5 min	· +	ND	ND	+ ()
Δ4	~ 120 min	~5 min	~5 min	+	+ .	÷	÷
Δ3	~ 120 min	~5 min	ND	+	+	÷	+
Δ2	15 -30 min	15 -30 min	15 -30 min	+	+/-		-
Δ1	15 -30 min	15 -30 min	15 -30 min	+	+/-	-	-

Table 4. Properties of IkBa C-terminal mutants.

Half life of $I\kappa B\alpha$ in the presence of either cycloheximide alone (*a*), in combination with TNF- α (*b*), or LPS (*c*) was determined by immunoblotting followed by densitometric analysis as shown in Figures 32 and 33. The ability of $I\kappa B\alpha$ mutants to associate with RelA(p65) *in vitro* (*d*) or *in vivo* (*e*) were taken from data presented in Figure 27 and Figure 31. Inhibition of NF- κB dependent transcription (*f*) and dissociation of NF- κB -DNA complexes (*g*) were taken from data presented in Figures 28 and 26, respectively.

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CAT activity was observed only when RelA was co-expressed and this activity was inhibited by excess wild type I κ B α expression (Figure 28). I κ B $\alpha(\Delta 4)$ and I κ B $\alpha(\Delta 3)$ expression also repressed NF- κ B dependent CAT activity, whereas I κ B $\alpha(\Delta 1)$ and I κ B $\alpha(\Delta 2)$ expression did not reduce NF- κ B dependent activity (Figure 28). These results demonstrated that I κ B $\alpha(\Delta 1)$ and I κ B $\alpha(\Delta 2)$ were unable to suppress ReIA dependent transcription, which correlates with their reduced ability to dissociate NF- κ B complexes *in vitro*.

Tetracycline control of IxBa expression. To analyze IxBa deletions in stably transformed cells. IxBa expressing NIH-3T3 cells were generated using the tetracycline responsive system (see Materials and Methods). The use of a system which allows repression of exogenous $I_{\kappa}B\alpha$ expression is ideal given the growth inhibitory activity of $l\kappa B\alpha$ (chapter III). Polyclonal tTA-I $\kappa B\alpha$ cells, cultured in the absence of tetracycline for 72h were examined for exogenous IxB α expression (Figure 29A); IxB α (wt), IxB α (DM), $I\kappa B\alpha(\Delta 1)$, $I\kappa B\alpha(\Delta 2)$, $I\kappa B\alpha(\Delta 3)$, and $I\kappa B\alpha(\Delta 4)$ proteins were detected with apparent molecular weights of 38, 38, 31, 32, 35 and 36 kDa, respectively (Figure 29A, lanes 2, 4, 6, 8, 10 and 12). Wild type human $I \kappa B \alpha$ was distinguished from the 37 kDa murine homologue, due to a slight molecular weight difference (Figure 29A, lanes 1 and 2). The human IxB α proteins were expressed in polyclonal populations at levels ranging from equivalent to the endogenous murine $I \kappa B \alpha (I \kappa B \alpha \Delta 4)$ to levels 20-50 fold higher than the endogenous IxBa (IxBa Δ 1). Exogenous IxBa levels were reduced when cells were cultured in the presence of tetracycline (1 µg/ml) for more than 24h (Figure 29A, lanes 1, 3, 5, 7, 9 and 11), although the degree of repression varied between cell lines. A representative analysis of selected individual clones of $I_{\kappa}B\alpha(wt)$ and $I_{\kappa}B\alpha\Delta 1$ clones is shown in Figure 29B (compare lanes 5 and 6, 7 and 8, 9 and 10).

A time course experiment was set up using tTA-I κ B $\alpha(\Delta 1)$ cells to examine the kinetics of exogenous I κ B α gene repression and activation by tetracycline. Immunoblot

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Figure 28. IxB α mediated repression of NF-xB dependent transcription. N-Tera-2 cells were co-transfected with pHIV CAT reporter plasmid (3 µg) along with the RelA(p65) expression plasmid CMV-p65 (3 µg) and various SVK3-based plasmids expressing wild type or mutant IxB α (9 µg) as indicated. At 30h after transfection, cells were stimulated with PMA and CAT activity was analyzed at 48h.

Figure 29. Tetracycline-responsive expression of human $I \ltimes B \alpha$ in NIH 3T3 cells. A. Human $I \ltimes B \alpha$ was detected by immunoblotting in extracts from polyclonal tTA-I $\ltimes B \alpha$ (wt) (lanes 1 and 2), tTA-I $\ltimes B \alpha$ (DM) (lanes 3 and 4), tTA-I $\ltimes B \alpha$ ($\Delta 1$) (lanes 5 and 6), tTA-I $\ltimes B \alpha$ ($\Delta 2$) (lanes 7 and 8), tTA-I $\ltimes B \alpha$ ($\Delta 3$) (lanes 9 and 10), tTA-I $\ltimes B \alpha$ ($\Delta 4$) (lanes 11 and 12) cells. tTA-I $\ltimes B \alpha$ cells were cultured in the presence (lanes 1, 3, 5, 7, 9 and 11) or absence (lanes 2, 4, 6, 8, 10, and 12) of tetracycline (1 µg/ml). Arrows indicate bands corresponding to endogenous murine I $\ltimes B \alpha$ and exogenous human I $\ltimes B \alpha$. B. Individual clones of tTA-I $\ltimes B \alpha$ ($\Delta 1$) (lanes 1-6) and tTA-I $\ltimes B \alpha$ (wt) (lanes 7 -10) were grown in the presence (lanes 1, 3, 5, 7 and 9) or absence (lanes 2, 4, 6, 8 and 10) of tetracycline (1 µg/ml). Bands corresponding to wtI $\ltimes B \alpha$ and I $\ltimes B \alpha$ ($\Delta 1$) are indicated.









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analysis revealed that 4h after addition of tetracycline (1 µg/ml) to the media, exogenous IxB $\alpha(\Delta 1)$ levels were decreased by 50% and only basal levels of IxB $\alpha(\Delta 1)$ subsisted 24h following tetracycline addition (Figure 30 A and B). Removal of tetracycline by reseeding cells in tetracycline free media, did not however result in a rapid gene reactivation: IxB $\alpha(\Delta 1)$ levels reached their maximum only 72h after tetracycline removal. This indicates that the tTA transactivator responded promptly to tetracycline and that tetracycline persisted for a relatively long period of time in cells.

Association of $I \kappa B \alpha$ with RelA in vivo. To determine if $I \kappa B \alpha$ mutants could also associate with RelA(p65) in vivo, co-immunoprecipitation studies were performed with anti-RelA and anti-IkBa antibodies (Figure 31). In tTA-3T3 cells, endogenous IkBa coprecipitated with anti-RelA antibody (Figure 31, lane 2) and reciprocally, RelA coprecipitated with anti-IxB α antibody (Figure 31, lane 4). IxB α peptide present in excess during $I\kappa B\alpha$ immunoprecipitation prevented subsequent RelA immunoprecipitation, demonstrating the specificity of antibody recognition (Figure 31, lane 5). In tTA- $I\kappa B\alpha(wt)$ cells, human $I\kappa B\alpha$ was detected following two sequential $I\kappa B\alpha$ immunoprecipitations, and migrated just above the murine $I_{\kappa}B\alpha$ band (Figure 31, lane 8), whereas in tTA-I κ Ba(Δ 1) cells, human I κ Ba(Δ 1) migrated below murine I κ Ba (Figure 31, lane 13). In both cell lines, murine and human IxBa were coimmunoprecipitated with anti-RelA antibody (Figure 31, lanes 7 and 12) and RelA was co-immunoprecipitated by anti-I κ B α (Figure 31, lanes 9 and 14). The reaction was specific since co-immunoprecipitation was abolished by the addition of excess $I_xB\alpha$ peptide (Figure 31, lanes 10 and 15). As with wtIxB α , the majority of IxB $\alpha(\Delta 3)$ and $I \times B \alpha(\Delta 4)$ present in tTA-IxB α cells were associated with RelA (Table 4). However, the amount of $I\kappa B\alpha(\Delta I)$ complexed to ReIA was significantly reduced compared with the total amount of $I\kappa B\alpha(\Delta 1)$ present in the cell (Figure 31, lanes 12 and 13). Similarly, only a small fraction of $I\kappa B\alpha(\Delta 2)$ was associated with RelA (Table 4). These results indicated

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Figure 30. The kinetics of repression and activation by tetracycline. tTA $I\kappa B\alpha(\Delta 1)$ cells maintained in the presence of tetracycline (1 µg/ml) were re-seeded in tetracycline free media (lanes 1 to 6). Conversely, tTA $I\kappa B\alpha(\Delta 1)$ cells maintained for a week in tetracycline free media were re-seeded in media containing 1 µg tetracycline per ml (lanes 7 to 12). Levels of $I\kappa B\alpha(\Delta 1)$ were measured by immunoblotting (B) at time 0, (lanes 1 and 7), 4h (lanes 2 and 8), 24h (lanes 3 and 9), 32h (lanes 4 and 10), 48h (lanes 5 and 11) and 72h (lanes 6 and 12) after tetracycline removal (lanes 1 to 6) or addition (lanes 7 to 12). The intensity of the band corresponding to $I\kappa B\alpha(\Delta 1)$ was measured by laser densitometry and values are represented graphically in panel A.



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Figure 31. In vivo association of IxB α with RelA. Polyclonal tTA-3T3 (lanes 1-5), tTA-IxB α (wt) (lanes 6-10), and tTA-IxB α (Δ 1) (lanes 11-15) were metabolically labeled with ³⁵S methionine for 1h. Cell lysates were immunoprecipitated with a RelA specific antibody (lanes 1, 2, 6, 7, 11, 12) or with an IxB α specific antibody (lanes 3, 4, 5, 8, 9, 10, 13, 14, 15). IxB α antibody recognition was competed by the addition of excess IxB α peptide (2 µg) to the reaction (lanes 5, 10 and 15). Immunoprecipitates were collected on protein-A sepharose beads, washed stringently and boiled in 1% SDS; 0,5% 2-mercaptoethanol. Supernatants were collected and immunoprecipitated again with RelA antibody (lanes 1, 4, 5, 6, 9, 10, 11, 14, and 15) or with IxB α antibody (lanes 2, 3, 7, 8, 12 and 13). Bands corresponding to RelA, murine IxB α , human IxB α (wt) and IxB α (Δ 1) are indicated.



that wtIxB α , IxB $\alpha(\Delta 4)$, IxB $\alpha(\Delta 3)$ and IxB $\alpha(DM)$ (S283A,T291A) were stably associated with RelA *in vivo*, whereas the interaction of IxB $\alpha(\Delta 1)$ and IxB $\alpha(\Delta 2)$ with RelA was unstable *in vivo*.

Inducer mediated degradation of I κ B α **.** To examine the effect of C-terminal deletions on inducer mediated degradation of $I\kappa B\alpha$, tTA-I $\kappa B\alpha$ cells were treated with TNF- α (5) ng/ml) or LPS (10 µg/ml) and were analyzed for IsBa expression by immunoblotting (Figure 32 and Table 4). Stimulation with TNF- α or LPS resulted in a rapid decrease of murine and human $I \times B \alpha$ 15 min after stimulation. This decrease peristed up to 60 min after stimulation and was followed by *de novo* synthesis of IxB α (Figure 32A and Table 4), as previously described (44,107,159,273). Inducer mediated degradation of endogenous IkBa in was observed in all tTA-IkBa expressing cells (Figure 32B-F). In response to either TNF- α or LPS stimulation, $I\kappa B\alpha(\Delta 4)$, $I\kappa B\alpha(\Delta 3)$ and $I\kappa B\alpha(DM)$ degraded rapidly within 15-30 min (Figure 32B, C, F, and Table 4), whereas $l \kappa B \alpha(\Delta 1)$ and $I \ltimes B \alpha(\Delta 2)$ did not undergo inducer mediated degradation (Figure 32D, E and Table 4). To avoid effects associated with overexpression of exogenous $I\kappa B\alpha$, $tTA-I\kappa B\alpha(\Delta 1)$ cells in the above experiments were cultured in the presence of tetracycline (0.1 µg/ml). which reduced $I\kappa B\alpha(\Delta I)$ expression to levels equivalent to endogenous murine $I\kappa B\alpha$. These experiments indicates that the region deleted in $I\kappa B\alpha(\Delta 2)$ but present in $I\kappa B\alpha(\Delta 3)$ - aa 269 to aa 287 - may play a role in TNF- α and LPS mediated degradation.

Intrinsic IxBa stability. Since inducer mediated degradation of IxBa does not require *de novo* protein synthesis (116,273), the turnover rate of IxBa in the absence (intrinsic stability) or presence of stimulus (inducer mediated degradation rate) was measured in cells treated with the protein synthesis inhibitor cycloheximide (50 µg/ml). tTA-3T3 and tTA-IxBa cells were stimulated with TNF-a for 2h and levels of IxBa were measured and quantified (Figure 33). The intrinsic stability of human wtIxBa was similar to that

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Figure 32. Inducer mediated degradation of $I \ltimes B \alpha$. Polyclonal tTA-I $\ltimes B \alpha$ (wt) (A), tTA-I $\ltimes B \alpha$ ($\Delta 4$) (B), tTA-I $\ltimes B \alpha$ ($\Delta 3$) (C), tTA-I $\ltimes B \alpha$ ($\Delta 2$) (D), tTA-I $\ltimes B \alpha$ ($\Delta 1$) (E) and tTA-I $\ltimes B \alpha$ (DM) (F) cells were stimulated with TNF- α for 0 (lane 1), 15 (lane 2), 30 (lane 3), 60 (lane 4), 90 (lane 5) and 120 min. (lane 6). Prior to stimulation, tTA-I $\ltimes B \alpha$ ($\Delta 1$) cells were cultured in the presence of tetracycline (0.1 µg/ml) to reduce the level of exogenous human I $\ltimes B \alpha$. Endogenous murine and exogenous human I $\ltimes B \alpha$ were detected in whole cell extracts (15 µg) by immunoblotting using affinity purified AR20 antibody.



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Figure 33. Analysis of $I\kappa B\alpha$ turnover rate. Polyclonal tTA-3T3 (A), tTA-I $\kappa B\alpha$ (wt) (B), tTA-I $\kappa B\alpha$ ($\Delta 4$) (C), tTA-I $\kappa B\alpha$ ($\Delta 3$) (D) and tTA-I $\kappa B\alpha$ ($\Delta 1$) (E) cells were treated with cycloheximide (50 µg/ml) alone (lanes 1-6) or stimulated with TNF- α (5 ng/ml) in the presence of cycloheximide (lanes 7-12) for 0 (lanes 1 and 7), 15 (lanes 2 and 8), 30 (lanes 3 and 9), 60 (lanes 4 and 10), 90 (lanes 5 and 11) and 120 min. (lanes 6 and 12). Endogenous murine and exogenous human I $\kappa B\alpha$ were detected in whole cell extracts (15 µg) by immunoblotting, using affinity purified AR20 antibody.

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of murine IkB α (Figure 33A, B, lanes 1-6): without inducer, both proteins had a half life of approximately 2h (summarized in Table 4). IkB $\alpha(\Delta 3)$, IkB $\alpha(\Delta 4)$ and IkB $\alpha(DM)$ also had intrinsic stabilities similar to wtIkB α (Figure 33, C and D, lanes 1-6; Table 4). However, the C-terminal deletion in IkB $\alpha(\Delta 1)$ (Figure 33E, lanes 1-6) and IkB $\alpha(\Delta 2)$ (Table 4) destabilized IkB α by reducing its half life to approximately 30 min. Following TNF- α stimulation in the presence of cycloheximide, the half life of wtIkB α , IkB $\alpha(DM)$, IkB $\alpha(\Delta 4)$, IkB $\alpha(\Delta 3)$, and murine IkB α was decreased to about 5 min (Figure 33A, B, C and D, lanes 7-12). In contrast, the degradation rate of IkB $\alpha(\Delta 2)$ and IkB $\alpha(\Delta 1)$ in the presence of TNF- α was similar to their respective intrinsic stabilities (Figure 33E, lanes 7-12; Table 4). These experiments demonstrate that deletion of the domain aa 269 to 287 desensitized IkB α to TNF- α mediated degradation and simultaneously accelerated IkB α turnover in unstimulated cells. Similar results were obtained when LPS was used as inducer (Table 4).

Protection of I_κBα **mutants by protease inhibitors.** To determine if the reduced half life of I_κBα(Δ 1) and I_κBα(Δ 2) mutants was related to inducer mediated degradation and the proteasome pathway, tTA-I_κBα(wt), tTA-I_κBα(Δ 1) and tTA-I_κBα(Δ 2) cells were pretreated for 1h with calpain inhibitor I or the MG132 proteasome inhibitor, both of which are known to block inducer mediated degradation of I_κBα (Figure 34). Cells were then treated with cycloheximide for 1h and the level of I_κBα remaining was determined by immunoblot and densitometric analysis. In the presence of cycloheximide, the amount of wtI_κBα remaining at 60 min was approximately 65-70% of the level at time 0; pretreatment of wtI_κBα expressing cells with protease inhibitors did not significantly increase the amount of remaining wtI_κBα in I_κBα(Δ 1) and I_κBα(Δ 2) expressing cells was reduced to 5-20% of the initial level, reflecting the rapid degradation of the Δ1 and Δ2 deletion mutants. However, in the presence of calpain inhibitor I or MG132, both

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Figure 34. Stabilization of mutant $l\kappa B\alpha$ by peptide aldehydes. tTA- $l\kappa B\alpha(wt)$. tTA- $l\kappa B\alpha(\Delta 1)$ and $l\kappa B\alpha(\Delta 2)$ expressing cells were treated for 1h with calpain inhibitor I (100 mM) or MG132 proteasome inhibitor (40 mM). Ethanol and dimethyl formamide, solvents for calpain inhibitor I and MG132 respectively, were added to the cells as controls. Cells were then treated with cycloheximide for 1h. The percentage of exogenous $l\kappa B\alpha$ remaining at the end of 1h cycloheximide treatment was determined by immunoblot analysis and compared to the amount of $l\kappa B\alpha$ at time 0. The amount of wt $l\kappa B\alpha$ (open bar), $l\kappa B\alpha(\Delta 2)$ (solid bar) and $l\kappa B\alpha(\Delta 1)$ (hatched bar) after 1h cycloheximide treatment is illustrated graphically. $I\kappa B\alpha(\Delta 1)$ and $I\kappa B\alpha(\Delta 2)$ were dramatically stabilized and did not degrade significantly during the period of cycloheximide treatment. These results imply that $I\kappa B\alpha(\Delta 1)$ and $I\kappa B\alpha(\Delta 2)$ have a reduced half life because they are constitutively degraded by proteases active during inducer mediated degradation.

Analysis of protein stability in $I\kappa B\alpha(2N\Delta 1)$ expressing cells. Substitution of serines 32 and 36 to alanine (S32A/S36A) were shown previously to protect $I\kappa B\alpha$ from inducer mediated degradation (42.43.272.300). To confirm that $I\kappa B\alpha(\Delta 1)$ and $I\kappa B\alpha(\Delta 2)$ are constitutively degraded by the same pathway responsible for inducer mediated degradation of $I\kappa B\alpha$, the substitutions S32A/S36A were introduced into $I\kappa B\alpha(2N\Delta 1)$ mutant. NIH 3T3 cells were stably trasfected with pSVK3 vector encoding $I\kappa B\alpha(2N\Delta 1)$. $I\kappa B\alpha(2N\Delta 1)$ expressing cells were treated with cycloheximide (50 µg/ml) and the turn over of $I\kappa B\alpha(2N\Delta 1)$ was measured by immunoblot (Figure 35B, lane 1 to 6). $I\kappa B\alpha(2N\Delta 1)$ was dramatically more stable than $I\kappa B\alpha(\Delta 1)$ (Figure 35B, lanes 1 to 6) and $I\kappa B\alpha(2N\Delta 1)$ stability was not affected by TNF- α treatment (Figure 35B, lanes 7 to 12). These results confirm that deletion of the C-terminus of $I\kappa B\alpha$ bypasses steps in the inducer-mediated degradation pathway, exposing $I\kappa B\alpha$ to the constitutive proteasome activity. Mutation S32A/S36A blocks $I\kappa B\alpha$ degradation, suggesting that in absence of the C-terminus, the N-terminal serines 32 and 36 are constitutively phosphorylated.

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Figure 35. Analysis of protein stability in $I\kappa B\alpha(2N\Delta 1)$ expressing cells. $I\kappa B\alpha(\Delta 1)$ expressing cells $[tTA-I\kappa B\alpha(\Delta 1)]$ (A) and cells stably transfected with $I\kappa B\alpha(2N\Delta 1)$ [SVK3/I $\kappa B\alpha(2N\Delta 1)$] (B) were treated with cycloheximide for 0 (lane 1 and 7), 15 (lane 2 and 8), 30 (lane 3 and 9), 60 (lane 4 and 10), 90 (lane 5 and 111), and 120 min (lane 6 and 12) alone (lane 1 to 6) or in combination with TNF- α (lane 6 to 12). Endogenous murine $I\kappa B\alpha$ (mI $\kappa B\alpha$), $I\kappa B\alpha(\Delta 1)$ and $I\kappa B\alpha(2N\Delta 1)$ were detected by immunoblot analysis using MAD 10B I $\kappa B\alpha$ monoclonal antibody.

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Chapter VI

Transdominant Mutants of IκBα Block Tat-TNF-α Synergistic Activation of HIV-1 Gene Expression and Virus Multiplication

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Previous studies have demonstrated that point mutation of N-terminal serines 32 and 36 to alanine protects $I\kappa B\alpha$ from inducer mediated degradation (42,43,272,300). Moreover, the study described in chapter IV, revealed that mutation of the casein kinase II sites within the C-terminus increased the half life of $I\kappa B\alpha$. Together these studies have identified the important regulatory phosphorylation sites in $I\kappa B\alpha$. Transdominant mutants of $I\kappa B\alpha$ were tested for their ability to repress the strong activation of the HIV-1 LTR by Tat and TNF- α .

Tat-TNF- α stimulation of the HIV-1 LTR. To determine the specific conditions required for Tat-TNF- α synergistic activation of HIV-1 LTR driven reporter constructs in Jurkat T cells, titration of wtTat expression plasmid and/or recombinant TNF- α was initially performed (data not shown). In subsequent experiments, Tat plasmid was used at a concentration of 2 µg, while TNF- α was used at a concentration of 5ng/ml. Tat stimulated the HIV-1 LTR CAT reporter plasmid approximately 10-fold while TNF- α treatment alone induced reporter gene expression about 5-fold (Figure 36A). Addition of both activators produced a true synergistic stimulation of the HIV-1 LTR, resulting in a 50-70 fold induction of gene expression (Figure 36A).

Tat-TNF- α synergism requires Tat binding to the TAR element. Previous studies demonstrated that efficient transactivation of HIV-1 gene expression by Tat required physical interaction between Tat and the TAR element (70). R52Q,R53Q point mutations of the HIV-1 Tat protein abrogated Tat binding to the TAR element and gene transactivation (70). To test whether the Tat-TNF- α synergism defined in the above model system required Tat-TAR association, the R52Q,R53Q mutant of Tat (designated TatRQ) was used together with TNF- α to stimulate expression of the HIV-1 LTR driven CAT reporter (Figure 36B). Whereas, Tat+TNF- α activated the HIV-1 LTR up to 70fold, TNF- α alone, wtTat alone or TatRQ alone induced LTR mediated gene expression

Figure 36. Tat-TNF- α activation of the HIV LTR requires Tat binding to the TAR element. Jurkat T cells were co-transfected with ptzIIICAT (5 µg) and 2 µg of pSVexTat (A) or Tat (R52Q,R53Q) (B) expression plasmids: cells were also transfected with 2 µg of LTR- Δ B (C) or LTR-B-ACU (D) and 2 µg of pSVexTat. At 32h after transfection, cells were incubated for an additional 16h in the presence or absence of TNF- α . CAT activities were assayed using whole cell extracts (50 µg for 30min). Cells transfected only with ptzIIICAT in the absence of activators were used as negative control. The results are the average of three experiments.

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4-, 6- and 3-fold respectively. Significantly, the combination of TNF- α treatment and TatRQ co-expression was only weakly effective (5-fold induction) in mediating activation of the HIV-1 LTR (Figure 36B). Similarly, mutations within the TAR element (ΔB and B-ACU) that altered the Tat protein binding site (70,239) were also not activated by Tat-TNF- α treatment (Figure 36C and 1D). These results thus reflect a requirement for Tat-TAR interactions in the synergistic transactivation of the HIV-1 LTR by Tat and TNF- α .

Tat-TNF- α synergism requires intact NF- κ B sites. To characterize the region of the HIV LTR involved in synergistic activation, HIV-1 LTR deletion mutants were transfected into Jurkat cells and stimulated with Tat, TNF- α or Tat+TNF- α . As expected, the intact LTR-CAT construct (plasmid ptzIIICAT) was strongly inducible by both activators (Figure 37), with an 18 fold induction with Tat+TNF- α . In contrast, the -109/-79 plasmid, a construct lacking the NF-kB sites, had only baseline level of activity and was not transactivated by TNF- α . Surprisingly, this construct was not inducible by wtTat expression plasmid, even though it contained an intact TAR element. Nevertheless, the combination of Tat and TNF- α was able to stimulate the -109/-79 plasmid about 5-fold. The III $\Delta 23$ construct which is deleted for the upstream modulatory sequences of the HIV-1 LTR (upstream of -167) was stimulated by both activators as efficiently as the wild LTR-CAT plasmid, indicating that the upstream elements in the -423 to -167 domain of the LTR did not play a complementary role in Tat-TNF- α activation. The plasmid III ΔA , deleted for all of the LTR sequences upstream of -57, was not activated significantly by either inducer alone, although a residual 4-fold induction of the III ΔA plasmid was observed with Tat-TNF- α . This experiment indicates that strong synergistic activation of the HIV-1 LTR by the combination of Tat and TNF- α required intact NF- κ B sites. The fact that some activation could occur in the absence of the enhancer element (in constructs -109/79 and

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Figure 37. Maximum Tat-TNF- α synergism requires intact NF- κ B sites. Jurkat T cells were co-transfected with 5 µg of ptzIIICAT, -109/-79, III Δ 23, or III Δ A in the absence or presence of pSVexTat (2 µg). TNF- α was added at 32h after transfection and incubated for an additional 16h. Whole cell extracts were prepared, normalized for total protein, and assayed for CAT activity (50 µg for 30min).

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IIIA) suggests that TNF- α may be able to potentiate Tat activity at the TAR element independently of the NF- κ B sites.

Stability of the $I \times B \alpha$ mutants. Recent experiments have defined specific sites of inducer mediated and constitutive phosphorylation in the IkBa regulatory protein (see Figure 25). In particular, mutation of the N-terminal phosphorylation sites S32 and S36 in the signal response domain of $I\kappa B\alpha$, prevented inducer mediated phosphorylation and subsequent proteasome-dependent degradation of $I \times B \alpha$ (42.43,52). Also, triple point mutation of $I\kappa B\alpha$ in the C-terminal residues S283, T291 and T299 abrogated constitutive phosphorvlation *in vivo* by casein kinase II and increased intrinsic stability of $I_{\kappa}B\alpha$, but did not affect inducer mediated degradation of $I \times B \alpha$ (165). Ix B α expression plasmids were generated that produced IkB α proteins singly mutated in S32, S36, in both S32 and S36 [IxB α (2N)], in the three C-terminal sites [IxB α (3C)] or in all five regulatory phosphorylation sites [IxB α (2N+3C)]. The inducer mediated turnover of these proteins was analyzed in stably transfected NIH 3T3 cells (21,165) at different times after treatment with cycloheximide and TNF- α (Figure 38A). Immunoblot analysis using an IkB α specific antibody was able to distinguish between the endogenous murine IkB α and the exogenously expressed human I κ B α (Figure 38A, lane 1). Cycloheximide was added to eliminate the complicating effects of de novo synthesis of $I \times B \alpha$ after induction. The endogenous murine $I\kappa B\alpha$, human wt $I\kappa B\alpha$ and $I\kappa B\alpha(3C)$ all degraded rapidly in response to TNF- α addition with a T1/2 of much less than 15 min (Figure 38A, lanes 2-6), indicating that triple mutation of S283, T291 and T299 did not affect inducer mediated degradation of $I \kappa B \alpha$. In contrast, both S36A (Figure 38A) and S32A mutations (data not shown) stabilized $I\kappa B\alpha$; both point mutations increased the T1/2 of IxBa to approximately 90-120 min (Figure 38A, lanes 2-6). Double point mutated $I\kappa B\alpha(2N)$ and $I\kappa B\alpha(2N+3C)$ were extremely stable in the presence of TNF- α and cycloheximide, with a T1/2 of greater than 4h (Figure 38B). This experiment

Figure 38. Inducer mediated degradation of $I\kappa B\alpha$. tTA-1 $\kappa B\alpha$ (wt), tTA-I $\kappa B\alpha$ (2N), tTA-I $\kappa B\alpha$ (S36A), tTA-I $\kappa B\alpha$ (3C) and tTA-I $\kappa B\alpha$ (2N+3C) cells were treated with TNF- α (5 ng/ml) and cycloheximide (50 µg/ml) for 0 (lane 1), 15 (lane 2), 60 (lane 3), 120 (lane 4), 180 (lane 5) or 240 min. (lane 6). (A) Endogenous murine and exogenous human I $\kappa B\alpha$ were detected in whole cell extracts (15 µg) by immunoblotting using affinity purified AR20 antibody. (B) Levels of I $\kappa B\alpha$ were quantified by laser densitometry and are represented graphically.

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demonstrates the increased stability of point mutated $I\kappa B\alpha$ molecules and suggests that both $I\kappa B\alpha(2N)$ and $I\kappa B\alpha(2N+3C)$ should be stable transdominant negative mutants of the NF- κ B response.

Inhibition of Tat-TNF- α activation of the HIV-1 LTR by $I_{\kappa}B\alpha$ transdominant mutants. To examine the effect of wild type and mutated forms of $I_{\kappa}B\alpha$ on Tat-TNF synergistic activation of HIV-1 LTR directed gene expression, the different forms of $I_{\kappa}B\alpha$ were co-transfected into Jurkat cells together with the reporter construct and the wtTat expression plasmid. Expression of both wild type $I_{\kappa}B\alpha$ and $I_{\kappa}B\alpha(3C)$ reduced Tat-TNF- α activation in a dose dependent manner from a level of about 70-fold transactivation to 15-30 fold induction (Figure 39). However, neither wtI $\kappa}B\alpha$ nor $I_{\kappa}B\alpha(3C)$ completely inhibited HIV-1 LTR mediated expression. Expression of the S32 mutant of $I_{\kappa}B\alpha$ (or S36) dramatically reduced Tat-TNF- α activation to 10-20 fold stimulation. Strikingly, the $I_{\kappa}B\alpha(2N)$ and $I_{\kappa}B\alpha(2N+3C)$ mutants were able to eliminate Tat-TNF- α transactivation at low concentrations of inhibitory plasmid; $I_{\kappa}B\alpha(2N)$ or $I_{\kappa}B\alpha(2N+3C)$ at 1 µg/ml reduced LTR-directed reporter gene expression to only 5-fold stimulation. At higher concentrations, Tat-TNF- α transactivation of HIV-1 LTR driven gene expression was completely suppressed by the transdominant $I_{\kappa}B\alpha$ mutants (Figure 39).

Inhibition of HIV-1 protein and RNA synthesis in a single cycle infection model. To examine the ability of the $I\kappa B\alpha$ mutant proteins to interfere with HIV-1 multiplication, HIV-1 proviral DNA (pSVC21 BH10) was transfected together with increasing amounts of wt $I\kappa B\alpha$ or mutant $I\kappa B\alpha$ -expressing plasmids into Cos-1 cells. This system permits a single round of virus multiplication and release of infectious HIV-1 but re-infection does not occur because of the absence of the CD4 receptor on Cos-1 cells. At three days post-transfection, high levels of transfected $I\kappa B\alpha$ accumulated in

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Figure 39. Transdominant IxB α mutants inhibit Tat-TNF- α activation of the HIV LTR. In Jurkat cells, pTZIIICAT (2 µg) was co-transfected with pSVexTat and different amounts (1, 2, 4 µg) of pSVK3/IxB α (wt), pSVK3/IxB α (S32), pSVK3/IxB α (2N) (S32A, S36A), pSVK3/IxB α (3C) (S283A, T291A, T299A), pSVK3/IxB α (2N+3C), and treated with TNF- α (5 ng/ml) for 16h beginning at 32h post-transfection. The level of HIV-1 LTR driven reporter gene expression was determined by CAT assay on the whole protein extracts of the cells. Positive control (+Tat/TNF- α) was obtained by co-transfection with HIV-1 LTR CAT (2 µg) and pSVexTat (2 µg), and induction with TNF- α (5 ng/ml). The negative control (-) was obtained by transfection with the HIV-1 LTR reporter construct only. The results represent the average of three independent experiments.

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Cos-1 cells (Figure 40B, lane 3-14) compared to the endogenous level of primate IkBa (Figure 40B, lanes 1 and 2). Using a human antiserum that recognizes HIV-1 structural proteins, a dramatic reduction in the amounts of viral specific p24 core antigen, p55 precursor for virion core proteins, and gp120 envelope glycoprotein was observed in cells expressing $I \kappa B \alpha(2N)$ (Figure 40A, lanes 6-8). In other experiments using the single cycle infection model, we found that wtIkB α , IkB α (3C) and IkB α (2N+3C) were also effective in blocking a round of HIV-1 replication, but $I\kappa B\alpha(2N)$ was consistently more effective in a dose dependent manner. Analysis of the intracellular accumulation of viral mRNA also confirmed that $I \times B \alpha$ and the transdominant negative forms of $I \times B \alpha$ differentially inhibited HIV-1 multiplication (Figure 41). Expression of $I\kappa B\alpha$ inhibited HIV-1 proviral transcription in a dose dependent manner (Figure 41A, lanes 3-14). In particular, $I\kappa B\alpha(2N)$ was the strongest inhibitor of HIV-1 transcript accumulation (Figure 41A, 6-8). Complementary results were obtained when the level of p24 antigen release into the supernatant was measured by an enzyme-linked immunosorption assay (ELISA)-based viral antigen capture assay (Figure 40D). Together these results indicate that $I \ltimes B \alpha(2N)$ inhibited HIV-1 transcript levels, intracellular viral protein accumulation and release of virions into the supernatant. Surprisingly, additional mutations within the C-terminal phosphorylation sites in $I\kappa B\alpha(2N+3C)$ reduced the inhibitory capacity relative to $I\kappa B\alpha(2N)$ (Figure 40A & D, lanes 12-14 and Figure 41A, lanes 12-14), suggesting an important role for the intact C-terminal PEST domain in the inhibition of HIV-1 multiplication.

Association of RelA with IxBa point mutants.

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Mutations in $I\kappa B\alpha(2N + 3C)$ may weaken the association with NF- κB proteins and thus affect HIV-1 replication in Cos-1 cells. NIH 3T3 cells were co-transfected with a plasmid encoding RelA (CMV-p65) and pSVK3-based plasmids encoding the various I $\kappa B\alpha$ point mutants. From these transfected cells, I $\kappa B\alpha$ was immunoprecipitated and

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Figure 40. Inhibition of viral protein expression by transdominant $I\kappa B\alpha$ mutants. Cos-1 cells were transfected with HIV-1 proviral DNA (pSVC21 BH10: 10 µg) and viral protein expression was inhibited by co-transfecting either 1 µg (lane 3, 6, 9, and 12), 5 µg (lane 4, 7, 10 and 13) or 10 µg (lane 5, 8, 11, and 14) of plasmid expressing wtI $\kappa B\alpha$ (lane 3-5), $I\kappa B\alpha(2N)$ (lane 6-8), $I\kappa B\alpha(3C)$ (lane 9-11) or $I\kappa B\alpha(2N+3C)$ (lane 12-14). Three days after transfection, cells were collected and analysed by immunoblotting for expression of HIV proteins (A). $I\kappa B\alpha$ (B) and β -actin (C). Bands corresponding to the viral envelope glycoprotein gp120, the p55 polyprotein precursor for virion core proteins , and the viral capsid protein p24 are indicated. D) The release of HIV-1 p24 antigen into the supernatant of infected cells was measured by p24 ELISA: the results represent the average of two independent experiments.

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Figure 41. Inhibition of viral transcription by transdominant $I \times B \alpha$ mutants. Cos-1 cells were transfected with HIV-1 proviral DNA (pSVC21 BH10: 10 µg) and either 1 µg (lane 3, 6, 9, and 12), 5 µg (lane 4, 7, 10 and 13) or 10 µg (lane 5, 8, 11, and 14) of plasmids expressing wild type I×B α (lane 3-5), I×B α (2N) (lane 6-8), I×B α (3C) (lane 9-11) or I×B α (2N+3C) (lane 12-14). Three days after transfection cells were collected and RNA was extracted. (A) Viral transcripts were detected by northern blot analysis; the HIV-1 proviral cDNA probes were the 2 and 2.2 kbp Hind-III fragments derived from pSVC21 BH10. Positions of the 9 kb, 4.5 kb and 2 kb transcripts are indicated. (B) The blot in (A) was stripped and reprobed with a β-actin probe to illustrate the relative amount of RNA present in each lane. The β-actin cDNA used as probe was the 1 kbp Pst-1 fragment derived from pβ-actin plasmid.



detected by immunoblotting using MAD 10B $I\kappa B\alpha$ monoclonal antibody. Endogenous murine $I\kappa B\alpha$ was distinguished from transfected $I\kappa B\alpha$ by its faster migration on SDS PAGE (Figure 42, lanes 1, 3, 5, 7, and 9). Similar amounts of transfected wild type $I\kappa B\alpha$, $I\kappa B\alpha(2N)$, $I\kappa B\alpha(3C)$ and $I\kappa B\alpha(2N + 3C)$ were detected by immunoblot from ReIA immunoprecipitates (Figure 42, lanes 4, 6, 8 and 10). This result demonstrates that the point mutations do not affect the capacity of $I\kappa B\alpha$ to physically associate with ReIA

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Figure 42. Association of RelA with $I \ltimes B \alpha$ point mutants. NIH 3T3 cells were co-transfected with 5 µg of CMV-p65 plasmid together with 5 µg of control pSVK3 plasmid (lanes 1 and 2), pSVK3/I \ltimes B α (wt) (lanes 3 and 4), SVK3/I κ B α (2N) (lanes 5 and 6), SVK3/I κ B α (3C) (lanes 7 and 8) or SVK3/I κ B α (2N + 3C) (lanes 9 and 10). At 40h after transfection, cells were lysed and immunoprecipitations were performed using polyclonal antibody directed against I κ B α (AR20) (lanes 1, 3, 5, 7 and 9) or RelA (AR28) (lanes 2, 4, 6, 8 and 10). The presence of I κ B α in the immunoprecipitates was detected by immunoblot using MAD 10B monoclonal antibody. Bands corresponding to endogenous murine I κ B α (mI κ B α) and transfected human I κ B α (hI κ B α) are indicated.

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Chapter VII

Discussion

Disruption of $I_{\kappa}B\alpha$ regulation by antisense RNA expression leads to malignant transformation. The production of antisense RNA as a strategy to block functional expression of target genes has been used extensively, although the mechanisms by which antisense RNA alters gene expression may vary widely (235). Duplex RNA structures may inhibit basic mechanisms such as RNA transport from the nucleus, translational initiation, RNA splicing, or polyadenylation. We have shown that antisense $I_{\kappa}B\alpha$ RNA overexpression in NIH 3T3 cells decreased the steady state levels of $IxB\alpha$ protein. altered NF-kB DNA binding and gene activity and, most importantly, induced malignant transformation as measured by increased saturation density, growth in soft agar and tumorigenicity in nude mice. Transformation appears to be specifically caused by $I\kappa B\alpha$ antisense RNA, since overexpression of $I\kappa B\gamma$ antisense did not induce transformation (Table 1). Furthermore, the biological phenotype of $I \times B \alpha$ overexpressing cells is consistent with a negative regulatory effect of IxBa on cellular growth. However, we can not rule out the possibility that $I\kappa B\alpha$ antisense RNA may act at a distinct level and alter cellular growth by an undetermined mechanism. In this regard, recent studies demonstrated that modified analogues of antisense molecules may stimulate expression of the Sp-1 transcription factor in a ReIA- dependent manner (217). Whether such a mechanism alters the growth phenotype of 3T3 cells has not been determined.

As demonstrated previously, the post-translational release of cytoplasmic NF- κ B proteins via degradation of the I κ B α molecule provides a general mechanism by which the convergence of different inductive signals may result in the rapid nuclear translocation of NF- κ B and activation of NF- κ B dependent genes (reviewed in 22 and 172). Furthermore, I κ B α transcription is stimulated by NF- κ B itself, particularly ReIA, due to the presence of NF- κ B sites within the I κ B α promoter. Association of newly synthesized I κ B with NF- κ B reduces the nuclear translocation of NF- κ B and restores cytoplasmic NF- κ B/I κ B complexes, ultimately resulting in decreased gene activation (44.77,159,273). Disruption of I κ B α expression by antisense RNA production may

interfere with the capacity of 1xB to associate with and autoregulate NF-xB activity. We suggest that 1xB α has a potential tumor suppressor activity and functions in the normal cell to control the cytoplasmic-nuclear partitioning of the NF-xB family of proto-oncogenes. In support of this idea, our experiments also demonstrate that 1xB α overexpression decreases the saturation density of 1xB α expressing clones and alters cellular morphology. The human 1xB α gene has been localized to chromosome 14q13, a region not previously associated with tumorigenicity (160).

Interestingly, the level of NF- κ B binding activity was quite low in uninduced $l\kappa B\alpha(S)$ and $l\kappa B\gamma(S)$ clones. After induction with TNF- α , the relative proportions of ReIA. NF κ B1/p50 and NF κ B2/p52 subunits shifted in $l\kappa B\alpha(S)$ and $l\kappa B\gamma(S)$ clones appeared to differ (PB, unpublished data), suggesting that subtle alterations in the relative abundance and/or availability of NF- κ B subunits may contribute to the observed phenotypic differences. Also, immunoprecipitation with $l\kappa B\alpha$ antibody followed by immunoblot analysis of the immunoprecipitated products demonstrated that $l\kappa B\alpha$ is complexed with murine ReIA (PB, unpublished data). Further analysis of protein-protein interactions and turnover of $l\kappa B$ proteins may reveal regulatory alterations among the members of the NF- κ B family.

Expression of $I\kappa B\gamma$ is non-existent in normal NIH 3T3 cells but has been previously demonstrated in other tissues including lymphoid cells, generated as a consequence of aternative splicing of the *nfkb1* gene (98,129). Ectopic expression of $I\kappa B\gamma$ did not alter 3T3 cell growth characteristics, nor did it appear to alter the inducibility of NF- κB binding activity. The potential association of $I\kappa B\gamma$ with other members of the NF- κB family is currently under investigation.

A role for NF- κ B in tumorigenicity has been recently confirmed using antisense technology. Antisense phosphorothioate oligonucleotides (APO) to RelA, but not to NF κ B1, were shown to reduce the ability of various transformed cell lines to grow in soft agar and adhere to the extracellular matrix (119). A fibrosectoma cell line

overexpressing antisense ReIA RNA in a dexamethasone-inducible fashion, formed tumors in nude mice. Interestingly, tuniors regressed when overexpression of antisense ReIA RNA was induced by dexamethasone treatment (119). ReIA appears to play a role in the tumorigenicity of various cell lines by regulating cell adhesion. APO to ReIA blocked adherence of dimethyl sulfoxide-differentiated HL-60 leukemia cells, stimulated with TPA. In the same cell line, APO to ReIA also blocked TPA-induced expression of the adhesion molecule CD11b (266). Moreover, in ovarian carcinoma cells, APO to ReIA inhibited expression of the urokinase-type plasminogen activator (uPA) (225), an important enzyme that controls cell growth and invasiveness (145). Supporting the results of chapter III, the gene encoding uPA contains three potential NF- κ B sites and in cells treated with OPA to I κ B α uPA expression is increased by 50% (225). The changes in cell morphology and increased invasiveness induced by I κ B α (AS) overexpression in NIH 3T3, may thus result from alteration of cell adherence.

Cells from patients with ataxia telangiectasia (AT) do not arrest DNA synthesis in response to ionizing radiation and are thus hypersensitive to ionizing radiation. Cells from AT patients also have abnormally high constitutive NF- κ B. Jung *et al* recently demonstrated that overexpression of I κ B α restored normal NF- κ B activity and corrected the radiation sensitivity and DNA synthesis defect in fibroblasts from AT patients (52). These results suggest that NF- κ B activity allows cells to overcome the growth arrest signal from ionizing radiation. The recent demonstration that I κ B α transcription is positively regulated by NF- κ B proteins and forms an autoregulatory loop (44,77,159,273), together with the present data, suggests that one critical growth regulatory gene affected by disruption of NF- κ B autoregulation may be I κ B α itself.

Overexpression of p50/65 chimeric protein in NIH 3T3 cells.

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Previous experiments demonstrate that a fusion protein consisting of the Rel homology domain of NFxB1 and the C-terminal of RelA possesses transactivating potential comparable to p50-p65 heterodimers *in vitro* (166). The p50/65 fusion protein strongly transactivates transcription of chimeric promoters containing either the HIV enhancer or the IFN β PRDII domain. Although *in vitro* activation of transcription by p50/65 is inhibited by addition of recombinant 1kB α , its DNA binding activity to a HIV enhancer probe is enhanced by 1kB α addition. Furthermore it was demonstrated in chapter III that p50/65 binds less strongly to 1kB α compared to ReIA. We therefore hypothesized that overexpression of p50/65 may be sufficient to induce NF-kB dependent gene expression. Overexpression of p50/65 was predicted to mimic (AS)1kB α mRNA overexpression and to transform NIH 3T3 cells; however, p50/65 had no effect on cell phenotype nor gene expression.

Casein kinase II phosphorylates $I \kappa B \alpha$ in the C-terminal PEST domain. A protein kinase activity isolated from Jurkat T cells that specifically interacted with and phosphorylated recombinant $I \kappa B \alpha$ was characterized in our laboratory. This protein kinase was identified as casein kinase II based on biochemical and immunological criteria. Deletion analysis of $I \kappa B \alpha$ localized the phosphorylation sites to the C-terminal PEST-like domain and point mutation analysis revealed that double mutation of residues T291 and S283 dramatically reduced phosphorylation by the kinase both *in vitro* and *in vivo*. The finding that CKII constitutively phosphorylates the C-terminus of $I \kappa B \alpha$ has been subsequently confirmed by other groups (17,184).

CKII is a highly conserved and ubiquitous protein kinase. More than 50 proteins are known to be phosphorylated by CKII. Furthermore, CKII activity is unusually high in transformed and proliferating cells and tissues; a rapid, transient increase in CKII occurs in a variety of cells exposed to mitogenic stimuli or to differentiation inducing signals (220). Studies in Saccharomyces demonstrated that the catalytic subunit of CKII is required for yeast cell growth (212). Conversely, phosphorylation of serine residues in c-Myb, c-ErbA, and c-fos by CKII may repress transcription of genes involved in metabolic regulation, cell growth, or differentiation. Mutation of CKII phosphorylation sites in these proteins was associated with their conversion to oncogenic proteins whereas mutation of CKII phosphorylation sites in the adenovirus E1A protein converted this oncogene to a transformation-defective protein (reviewed in 187).

Recently, CKII α transgenic mice were created that developed lymphoproliferative disorders resembling the fatal lymphoproliferative syndrome associated with the protozoan parasite *Theileria parva* in cattle. Interestingly, theileriosis in cattle is characterized by the overexpression of casein kinase II. In the murine transgenic model, CKII α expressing mice displayed a stochastic potential to develop lymphoma; co-expression of a *c-myc* transgene in addition to CKII α resulted in neonatal leukemia. Based on this study, casein kinase II can serve as an oncogene, and its dysregulated expression is capable of transforming lymphocytes in cooperation with *c-myc* (262)

There are several potential sites of CKII serine/threonine phosphorylation in I κ B α (consensus S/T-X-X-E/D), including residues S32, S36, S283, T291, T299, Interestingly, the glutamic or aspartic acid residues at position 4 of the consensus sequence may be substituted by any phosphoamino acid. Thus, the residues S288, T296 and S293 can also serve as potential CKII phosphorylation sites when residues T291, T299 or T296 respectively, are phosphorylated. This possibility is if the schematically in Figure 43. Deletion analysis of $I\kappa B\alpha$ located the *in vitro* phosphorylation sites to the C-terminal PEST-like domain. Two critical sites (residues T291 and S283) were further identified by point mutation analysis. The fact that mutation T291A reduced phosphorylation by 2-4 fold, while other single point mutations had little or no effect, suggested that residue T291 was a critical

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CKII consensus: S/T-X-X-Acidic group

Figure 43. Schematic representation of human $I_xB\alpha$ and C-terminal casein kinase II phosphorylation sites. Human $I_xB\alpha$ contains five internal ankyrin repeats (SWI6/ANK) involved in the binding to NF- κ B molecules. The N-terminal region of IxB α between aa 20 to 45 is expanded above the schematic to show the one letter amino acid sequence. This region includes, the phosphorylation sites (S32 and S36), shown previously to play a role in inducer mediated degradation (42, 43), and the lysines 21 and 22 on which ubiquitination was shown to occur (14, 250). A region rich in proline, serine, threonine and glutamic acid, the PEST domain, spans aa 264 to 317; the C-terminal region of IxB α between aa 251 and 317 is expanded below the schematic to show the one letter amino acid sequence. The PEST domain contains several CKII phosphorylation sites clustered between aa283 and 302 at S283, T291 and T299 (underlined). Phosphorylation of residues T291 and T299 also generates new potential CKII phosphorylation sites at residues S288, T296, and S293 (denoted by lines and asterisks above the sequence).

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. سېرې phosphorylation site for CKII. The results of phosphoamino acid analysis *in vitro* supported this conclusion (165). In wt1xB α protein, the phosphothreonine/ phosphoserine ratio was 4:1, while in IkB α (T291A), the ratio was inversed 1:4. When S283 alone was changed to alanine, there was no effect on the phosphorylation by CKII *in vitro*; however, the double mutation (T291A,S283A) reduced phosphorylation more than 10-20 fold. Combined with the fact that the T291A mutation only reduced phosphorylation by 2-4 fold, the results indicate that residue 283 is also an important site for CKII phosphorylation. It is possible that threonine 291 and/or serine 283 may be responsible for positioning the kinase such that ordered series of phosphorylation events can occur, perhaps with T291 serving as an initial binding site for CKII.

CKII phosphorylated I κ B α both *in vitro* and *in vivo*. Many protein kinases can phosphorylate I κ B family proteins and activate NF- κ B/I κ B α complexes in cell-free systems (11). These kinases include protein kinase C (101), cAMP-dependent protein kinase (143). heme-regulated eIF-2 kinase (101), Raf-1 kinase (85), PKR (150), and a recently discovered multisubunit ubiquitin-dependent kinase (53). The latter kinase is particularly interesting since it phosphorylates the critical N-terminal residues S32 and S36 *in vitro* (53). Phosphorylation of I κ B α in intact cells was detected following treatment of cells with TNF- α , lipopolysaccharide, or phorbol 12-myristate 13-acetate (23,44,62,274). However, none of the kinases identified to date has been shown to directly phophorylate I κ B α *in vivo*.

In chapter IV, we identified *in vivo* sites of phosphorylation in $I\kappa B\alpha$ located in the Cterminal PEST domain. It was previously shown that $I\kappa B\alpha$ was constitutively phosphorylated in unstimulated cells and became hyperphosphorylated upon cell stimulation (43,73). Here we show that the constitutive phosphorylation of $I\kappa B\alpha$ is eliminated by mutation at residues T291, S283 and T299 [I $\kappa B\alpha$ (3C)]. Mutation of specific serine residues in the C-terminal PEST domain of the avian I $\kappa B\alpha$ (pp40) protein also eliminated phosphorylation of pp40 *in vivo* (244). Since the I κ B α (3C) mutation also eliminated phosphorylation of I κ B α by recombinant CKII *in vitro*, CKII appears to be the only protein kinase identified to date that can directly phosphorylate I κ B α *in vivo*. After stimulation of cells with TNF- α , the hyperphosphorylated form of wtI κ B α was detected by immunoblotting (Figure 23); however, phosphorylation of I κ B α (S283A, T291A) was reduced about 10 fold (Figure 23), suggesting that an intact C-terminal domain may also contribute to the generation of the hyperphosphorylated form of I κ B α .

Phosphorylation of I κ B α by CKII may alter its intrinsic stability. Since many eukaryotic proteins with a short half-life have PEST sequences and are phosphorylated by CKII, it has been suggested that phosphorylation may affect protein turnover (187). In both stably transfected cells and in transiently transfected cells, the half life of I κ B α in the presence of cycloheximide was increased by the three CKII site mutations. In stably transfected cells the half life of wtI κ B α was about 1h compared to 2h for the I κ B α (3C) mutant, while in transiently transfected cells, the half life was about 30 min for the wt protein compared to 90 min for the I κ B α (3C) mutant. These results indicates that the constitutive phosphorylation of I κ B α by casein kinase II plays a role in the constitutive turn over of the protein. Supporting this conclusion, studies by McElhinny *et al.* demonstrated that immunodepletion of CKII stabilizes I κ B α in an *in vitro* degradation assay (184).

IxB α phosphorylation by CKII is not directly linked with inducer mediated degradation. Recent studies by Brown *et al.* demonstrated that residues S32 or S36 were required for TNF- α mediated phosphorylation and degradation of IxB α (43), while Brockman *et al.* further showed that S32A or S32A/S36A substitutions fully protected IxB α from HTLV-1 Tax mediated degradation (42). Furthermore, Chen *et al.* demonstrated that S32A/S36A substitutions and to a lesser extent the S32A substitution.

prevented TNF- α induced ubiquitination of IkB α (52). These studies thus support a model in which IxBa is phosphorylated on residues S32 and/or S36 in response to multiple inducers and these phosphorylation events target $I\kappa B\alpha$ for ubiquitination and subsequent degradation by the proteasome (52). Both Ser-32 and Ser-36 are positioned within potential phosphorylation sites for CKII (Figure 43); however, it has not been possible to demonstrate a role for CKII phosphorylation in the N-terminal, signal response domain (RL, unpublished data). The fact that mutation of C-terminal CKII phosphorylation sites blocked completely constitutive phosphorylation of $I\kappa B\alpha$ in vivo suggests that these two potential amino-terminal sites are not phosphorylated by CKII in vivo. Nonetheless, C-terminal CKII sites may potentiate inducer-mediated hyperphosphorylation at the N-terminus since mutation of S283 and T291 greatly reduced the appearence of hyperphosphorylated $I\kappa B\alpha$ forms following TNF- α stimulation. The potentiation mediated by the C-terminal CKII sites however, is not required for inducer-mediated degradation of $I\kappa B\alpha$ since like wtI $\kappa B\alpha$, $I\kappa B\alpha(3C)$ is rapidly degraded in response to TNF- α induction. These results indicate that IxB α phosphorylation by CKII may not be required for inducer-mediated degradation, but rather plays a role in the constitutive turnover of the protein.

The role of the C-terminal domain of $I \ltimes B \alpha$ in protein degradation and stabilization. In Chapter V, we examined the biochemical and functional properties of C-terminal deletions in $I \ltimes B \alpha$ with respect to intrinsic protein stability, inducer mediated degradation, dissociation of NF- κ B-DNA complexes and association with RelA(p65)*in vitro* and *in vivo*. Our results demonstrate that: 1) the C-terminal end of I κ B α from aa 288 to aa 317 which includes most of the PEST domain is apparently dispensable for function since I κ B α (Δ 3) and I κ B α (Δ 4) behave like wtI κ B α ; 2) deletion of the region between aa 269 and aa 287 (I κ B $\alpha\Delta$ 2) abolishes responsiveness to TNF- α and LPS mediated degradation; 3) I κ B $\alpha\Delta$ 1 and I κ B $\alpha\Delta$ 2 mutants have a reduced intrinsic stability (T1/2 15-30 min) and are constitutively degraded by proteases that are inhibited by calpain inhibitor I and MG132; and 4) the domain between aa 269 and aa 287 is required for dissociation of NF- κ B-DNA complexes *in vitro*, for strong interaction with RelA *in vivo* and for efficient repression of NF- κ B dependent transcription.

In addition to the N-terminal serines 32 and 36, sequences within the C-terminus of IxB α also play a role in inducer mediated degradation (43,231,300). Whiteside et al. demonstrated that IkB α deletion from aa 279 to aa 317 abolished TNF- α and LPS mediated degradation (300). Our deletion studies also demonstrate that $I\kappa B\alpha$ deleted from aa 269 to aa 317 ($I\kappa B\alpha \Delta 2$) had a similar phenotype, whereas $I\kappa B\alpha$ deleted from aa 288 to as 317 had a phenotype indistinguishable from wtIxB α , as measured in several functional assays. Therefore, based on these two studies, a C-terminal domain involved in IxB α degradation is located between aa 279 and aa 287; the sequence MLPESEDEE (outlined in bold in Figure 25). This sequence contains one of the constitutive casein kinase II phosphorylation sites SEDE identified in chapter IV, but mutation of the S283 and T291 sites did not affect IkBa activity. Residues E284, D285 or E286 were previously identified as being critical for the dissociation of NF-xB-DNA complexes (80). Given the number of acidic amino acids in this short segment, it appears that the functional activity of this part of the C-terminal domain relates to its highly acidic nature. In fact, as shown in chapter IV triple mutation of S283, T291 and T299 increases the intrinsic stability of $I_x B\alpha$ (165).

Deletion of virtually the entire PEST domain in $I\kappa B\alpha(\Delta 3)$ did not alter $I\kappa B\alpha$ intrinsic stability or responsiveness to inducer mediated degradation, since this mutant behaved like wt I $\kappa B\alpha$ in several biochemical and functional assays (summarized in Table 4). However, deletion of the region adjacent to the PEST domain in $I\kappa B\alpha(\Delta 1)$ and $I\kappa B\alpha(\Delta 2)$ did not increase intrinsic protein stability, but rather decreased $I\kappa B\alpha$ stability from T1/2 ~120 min to T1/2~15-30 min. Chen *et al.* observed that $I\kappa B\alpha$ deletion $\Delta 243-317$ also did not undergo inducer mediated degradation, had a reduced half life, and did not interact

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with ReIA (52). Since free IkB α has a shorter half life than ReIA-bound IkB α (226), it was suggested that it is the absence of ReIA interaction *per se* which caused the Δ 243-317 mutant to undergo signal independent degradation (52). However, Rodriguez *et al* showed that an internal deletion from an 257-264 does not prevent inducer mediated degradation, even though it abolishes interaction with ReIA *in vivo* and *in vitro* (231). This aspect of IkB α activity is reminiscent of Cactus, a drosophila IkB homologue, that does not require Dorsal (NF-kB like) interaction to undergo inducer mediated degradation (27). Therefore the reduced ability of IkB α (Δ 1) and IkB α (Δ 2) mutants to bind ReIA is probably only one of the factors which contribute to their short half life and their unresponsiveness to inducer mediated degradation.

Based on our observations and the recent study of Sachdev *et al.* demonstrating that C-terminal mutations in chicken $I\kappa B\alpha(pp40)$ decreased the interaction with ReIA (244), we conclude that the region of $I\kappa B\alpha$ from au 269 to au 287 may strengthen interaction with p65 *in vivo*. Biochemical characterization of the domain structure of $I\kappa B\alpha$ demonstrated that $I\kappa B\alpha$ contains a highly structured central domain that is resistant to proteolysis and flexible N- and C-terminal extensions that are sensitive to proteolytic digestion (134). The C-terminal region was protected from proteolysis up to au 275 when $I\kappa B\alpha$ was bound to p65, suggesting that this region directly interfaced with p65 and was thus masked in the $I\kappa B\alpha$ -p65 complex.

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Inducer mediated degradation is inhibited by peptidyl aldehydes such as MG132 and calpain inhibitor I (52,168). In this study, we show that these inhibitors also dramatically increased the intrinsic stability of mutant $I\kappa B\alpha(\Delta 1)$ and $I\kappa B\alpha(\Delta 2)$ but not wt I $\kappa B\alpha$. Furthermore, substitution of serine 32 and 36 to alanine in $I\kappa B\alpha(\Delta 1)$ restores the wild type intrinsic stability and Chen *et al.* showed that deletion of the C-terminus did not prevent I $\kappa B\alpha$ ubiquitination since $\Delta 243-317$ mutant could still be ubiquitinated *in vitro* (52). We therefore propose that in unstimulated cells, the C-terminus protects I $\kappa B\alpha$ from constitutive proteasome mediated degradation. Upon stimulation, phosphorylation at the N-terminus abolishes protection by the C-terminus and targets $I\kappa B\alpha$ for ubiquitination and degradation. In absence of the C-terminus, residues S32 and S36 are presumably constitutively phosphorylated and thus $I\kappa B\alpha$ is targeted to the ubiquitindependent proteasome pathway of degradation. A model for inducer-mediated degradation of $I\kappa B\alpha$ is presented on Figure 44.

In view of the predominantly cytoplasmic localization of $1\kappa B\alpha$, the biological significance of NF- κ B-DNA complex dissociation by $1\kappa B\alpha$ is not yet understood, although $1\kappa B\alpha$ has previously been found in the nucleus (64). Furthermore, *in vitro* transcription studies using purified NF- κ B proteins demonstrated that addition of recombinant $1\kappa B\alpha$ to the transcription reactions inhibited NF- κ B dependent transcription (166.309). These experiments suggest that a novel nuclear role for newly synthesized $1\kappa B\alpha$ may be to directly inhibit NF- κ B dependent gene expression by dissociating NF- κ B-DNA transcription complexes. This idea is supported by the recent observation that following induction, *de novo* synthesized $1\kappa B\alpha$ protein transcription (7).

Transdominant mutants of I κ B α block Tat-TNF- α synergistic activation of HIV-1 gene expression and virus multiplication. In chapter VI, we examined the ability of different forms of I κ B α , mutated in distinct regulatory phosphorylation sites, to inhibit the the Tat-TNF- α synergistic activation of the HIV-LTR in Jurkat cells. We found in our Jurkat model that transactivation of the HIV LTR was dependent upon both functional Tat-TAR interaction and the presence of NF- κ B binding sites in the -100 enhancer region of the HIV LTR. Co-expression of wtI κ B α or mutant I κ B α inhibited Tat-TNF- α synergism in a dose dependent manner. Interestingly, the transdominant mutants I κ B α (2N) or I κ B α (2N+3C) were at least five times more effective than wtI κ B α in inhibiting HIV LTR directed gene expression. Moreover, I κ B α (2N) but surprisingly not I κ B α (2N+3C) was more effective in blocking HIV-1 protein and RNA synthesis in a

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Figure 44. A model of inducer mediated degradation of $I \ltimes B \alpha$. In unstimulated cells, $I \ltimes B \alpha$ may acquire a conformation permitting interaction between the N- and C-termini (A). Contact between the ankyrin repeat domains and the nuclear localization sequence maintains the NF- κ B/I κ B heterotrimer in the cytoplasm (A to D). Casein kinase II (CKII) constitutively phosphorylates the $I \ltimes B \alpha$ C-terminus and thereby contributes to the short half life of the protein. Following TNF- α or LPS stimulation, $I \ltimes B \alpha$ is phosphorylated on N-terminal residues S32 and S36 by an as yet unidentified protein kinase (B). The resulting net negative charge of the domains may modify $I \ltimes B \alpha$ structure, forcing the protein to adopt a new conformation (C) which can be recognized by the ubiquitin-conjugating enzymes, permitting ubiquitination on lysine 21 and 22. Following ubiquitination (D), $I \ltimes B \alpha$ is degraded by the proteasome (E). The NF- κB RelA-NF $\kappa B 1/p50$ heterodimer then translocate to the nucleus and bind DNA (D).

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single cycle infection model than wtIxB α or IxB α (3C). The observation that mutations in the C-terminal PEST domain of IxB α decreased the inhibitory potential of IxB α (2N) is surprising and indicates that an intact C-terminus is required for maximal inhibition of HIV-1 multiplication by IxB α (2N). This effect of the C-terminal domain was not apparent in assays measuring inhibition of LTR transactivation and may thus reflect a distinct functional activity for the IxB α C-terminus. Substitution of serine 283 and threonine 291 and 299 however appears not to affect association with NF- κ B as shown in co-transfection experiments. These experiments suggest a strategy that may contribute to inhibition of HIV-1 gene expression by interfering with the NF- κ B/Rel signaling pathway.

Our results are complementary to a number of recent experiments which have addressed the role of NF-kB transcription factors in HIV-1 regulated gene expression. Westendorp et al. demonstrated that HIV-1 Tat protein amplified the activity of TNF- α with regard to TNF- α induced activation of NF- κ B and TNF- α mediated cytotoxicity via the formation of reactive oxygen intermediates (295). Tat suppressed the expression of Mn-dependent superoxide dismutase (Mn-SOD) which normally functions as part of the cellular response to oxidative stress, thus shifting the cellular redox state towards prooxidative conditions. Under these conditions, higher levels of NF-xB binding activity that contribute to stimulation of HIV-1 LTR directed gene expression were observed (MC, unpublished data). Under conditions of maximal Tat-TNF-a synergism, IxBa was nonetheless able to interfere with NF- κ B induction, by sequestering NF- κ B in the cytoplasm in a concentration dependent manner (PB, unpublished data). $l_{\kappa}B\alpha$ molecules mutated in the N-terminal signal response phosphorylation sites S32 and/or S36 did not undergo rapid inducer mediated degradation (see Figure 38) and were at least five times more effective in blocking LTR-directed gene expression. Our experiments furthermore extend a recent study demonstrating that IxB molecules inhibited Tat-mediated transactivation of the HIV-LTR (110).

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Biswas *et al.* showed that Tat provided a low level of activation of the viral LTR, even in absence of a functional TAR element (30.31), thus confirming the earlier described TAR-independent mode of Tat action (3,13,278). The TAR-independent mode of Tat action was proposed to occur through the transcriptional activation of TNF- α which would in turn stimulate NF- κ B binding activity (31,295). These observations are reminiscent of a study demonstrating that interleukin-2 (IL-2) secretion was upregulated at the transcriptional level by the addition of extracellular Tat to activated T cells. The response element in the IL-2 promoter also mapped to the NF- κ B site at positions -206 to -195 (296). As was observed in the present study, Biswas *et al.* found that mutations in the NF- κ B motifs decreased Tat activation dramatically, indicating that maximal stimulation of the LTR directed gene expression required Tat-TNF cooperation (31).

A systematic comparison of HIV-1 LTR activity in human CD4 primary T cells and a transformed lymphoblastoid cell line J-Jhan was performed and strikingly different requirements for maximal LTR activation were observed (3). In unstimulated CD4 T lymphocytes, a low basal level of LTR activity was detected, whereas in the lymphoblastoid cell line a high spontaneous level of LTR activity was found that was essentially independent of the NF-kB responsive elements. In contrast, in primary lymphocytes there was an absolute dependence upon the NF-kB sites for initiation and Tat-mediated amplification of HIV-1 transcription. These results are in keeping with differences in the permissiveness to HIV replication of primary versus established cell types. In lymphoblastoid cell lines, HIV infection resulted in active replication in the absence of other stimuli, whereas in primary T cells, replication was undetectable and was dependent upon T cell activation for triggering of viral replication (3). In the present study we also found that HIV LTR-directed gene activity required both Tat-TAR transactivation and NF-kB induction, indicating that our Jurkat cell model may reflect more closely the quiescent state of primary T cells, rather than the activated state of other lymphoblastoid cell lines.

All of the above studies suggest a transcriptional role for $1\kappa B\alpha$ in the inhibition of HIV-1 LTR driven gene expression, consistent with the sequestration of NF- κB subunits in the cytoplasm. A recent study from Wu *et al.* however, implicated $1\kappa B\alpha$ at a distinct level in the HIV-1 life cycle (304). Using wtI $\kappa B\alpha$, HIV-1 replication was blocked at the post-transcriptional level of Rev function, not at a transcriptional level. Because $1\kappa B\alpha$ did not interact directly with Rev and NF- κB expression vectors potentiated Rev stimulation, it was concluded that NF- $\kappa B/1\kappa B$ regulated a cellular factor required for Rev function (304). Recent experiments have demonstrated that Rev contains an RNA binding domain, required for interaction with HIV-1 RNA and an effector domain required for RNA bound Rev to function. The Rev effector domain interacted specifically with host proteins with homology to nucleoporins, a class of proteins that mediate nucleocytoplasmic transport (35.93.269). It is possible that a novel function of $1\kappa B\alpha$ may be to interfere with Rev-mediated nuclear export of viral structural mRNA.

HIV-1 infection causes constitutive activation of NF-κB DNA-binding activity in infected cells (238). A direct temporal correlation exists between HIV infection and the appearance of NF-κB DNA-binding activity in myeloid cells (236,237), which may in turn prime or stimulate cytokine release. Cytokine release from HIV-1 infected cells may contribute to the elevated levels of TNF- α , IL-1, IL-6, TGF β and IFN γ , present in the sera of AIDS patients in late stage disease (reviewed in 238). Elevated IFN α/β activity is also present in the sera of AIDS patients in late stage disease and serves as a marker for poor prognosis (238). Expression of an IκB α transdominant may thus interfere with HIV-1 infection at multiple levels: at the level of NF- κ B dependent transcription activation of the LTR; at the level of Rev post-transcriptional activity; and at the level of expression of HIV-1 induced inflammatory cytokines.

Contributions to Original Knowledge

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The present studies on the mechanisms controlling $I\kappa B\alpha$ activity and function have contributed to a better understanding of $I\kappa B\alpha$ regulation of NF- κB activity, cell growth control and HIV-1 gene expression. The candidate's major contributions to original knowledge are listed below:

1. For the first time, $I\kappa B\alpha$ was specifically inactivated using anti-sense oligonucleotide technology. This study demonstrated that $I\kappa B\alpha$ plays an important role in cell growth since overexpression of anti-sense $I\kappa B\alpha$ mRNA transformed NIH 3T3 cells. The results also demonstrated that down-regulation of $I\kappa B\alpha$ alone leads to NF- κB activation.

2. Analysis of the C-terminal point mutants revealed that the casein kinase II (CKII) sites located in the $I\kappa B\alpha$ C-terminal PEST domain are constitutively phosphorylated *in vivo* and contribute to the appearance of hyperphosphorylated $I\kappa B\alpha$ forms following TNF- α stimulation. Mutation of these sites do not protect $I\kappa B\alpha$ from inducer-mediated degradation but increase the intrinsic stability of the protein demonstrating a role for CKII in $I\kappa B\alpha$ regulation.

3. Analysis of the C-terminal deletion mutants revealed that a region N-terminal of $I \times B \alpha$ PEST domain was important for the stability of the protein. Deletion of this region prevented $I \times B \alpha$ interaction with RelA *in vivo* and dramatically reduced protein stability. These results also revealed that this domain protected $I \times B \alpha$ from constitutive proteasome activity.

4. Examination of the transdominant negative phenotype of $I \ltimes B \alpha$ point mutants revealed that a mutant in which residues S32 and S36 are substituted to alanine efficiently repressed transcription from the HIV-1 LTR in cells stimulated with both TNF- α and HIV-1 Tat and also inhibited HIV-1 multiplication. Mutations of the Cterminal casein kinase II sites in addition to substitutions of residues S32 and S36 to alanine impaired the ability of $I \ltimes B \alpha$ to repress viral expression and/or replication in HIV-1 infected cells. This study indicates that $I \ltimes B \alpha$ may regulate HIV-1 multiplication at an additional level, thus opening a new avenue for future research. Chapter VIII

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