Molecular Analysis of NF-κB Activation in HIV-1 Infected Myeloid Cells

by

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To my family and relatives,
for all your support and encouragement which spurred me forward

To my friends,
for the laughter and fun which helped to keep me sane

To George,
for your strength and love which carried me through my most difficult moments

Abstract

Macrophages are important targets for HIV-1 infection, they are generally the first cells infected and are responsible for the extensive viral dissemination seen in HIV-1 infection. Monocytic cells are long lived and become ferocious producers of virus upon pathogen costimulation. This high level of viral replication may result from the activation of NF-κB transcription factors. In addition to orchestrating the host inflammatory response, NF-κB strongly transactivates the HIV-1 promoter, making NF-κB activation a double edged sword; stimuli that induce an NF-κB mediated response also lead to HIV-1 transcription.

We have used two promonocytic cell models to investigate the regulation of NF-κB signaling and its functional role in HIV-1 infected myeloid cells. Experiments conducted in U937 and PLB-985 cells and their HIV-1 infected counterparts U9-IIIB and PLB-IIIB, revealed that HIV-1 infected cells express constitutive NF-κB·DNA binding activity due to altered regulation of upstream kinases. The double stranded RNA dependent protein kinase (PKR) and the IκB kinase (IKK) are constitutively activated in these cells and lead to the phosphorylation and subsequent degradation of the inhibitory IκB proteins. Degradation of IκBα and IκBβ releases NF-κB which translocates to the nucleus and activates responsive genes, including κB dependent NF-κB genes p105/p50, p100/p52 and c-Rel. These NF-κB subunits are increased in HIV-1 infected U9-IIIB and PLB-IIIB cells and may contribute to constitutive NF-κB activation. In addition, IκBβ is able to enter the nucleus and bind DNA bound NF-κB forming a ternary complex. IκBβ shields NF-κB from dissociation by IκBα, maintaining transcriptionally active NF-κB. Degradation of IκBα and IκBβ therefore releases active NF-κB, and IκBβ additionally protects DNA bound NF-κB from inhibition from newly synthesized IκBα.

We further examined the function of constitutive NF-κB activation and found it to be important for maintaining cell viability of HIV-1 infected cells. TNFα induced apoptosis inversely correlated with NF-κB activation in monocytic cells and inhibition of constitutive NF-κB activation resulted in apoptotic death in HIV-1 infected, but not uninfected cells. Therefore, it seems that HIV-1 induces NF-κB activation by modulating upstream kinases so that the cell can resist HIV-1 induced apoptosis. These results may have important implications for the treatment of HIV-1 infection.

Resumé

Les macrophages représentent une cible importante de l'infection par le VIH: ce sont généralement les premières cellules infectées et ils sont responsables de la dissémination virale extensive qui a lieu au cours de l'infection. Les monocytes ont une longue durée de vie et produisent de grandes quantités de virus lors d'une seconde stimulation par un pathogène. Ce haut niveau de réplication virale est causé par l'activation des facteurs de transcription NF-κB, qui en plus de participer à la réponse inflammatoire de l'hôte transactivent fortement le promoteur du VIH. L'activation de NF-κB devient donc une arme à double tranchant: les stimuli qui induisent une réponse au cours de laquelle NF-κB est activé mènent aussi à une augmentation de la transcription du VIH.

Nous avons utilisé deux lignées de cellules promonocytaires comme modèle pour étudier la régulation de l'activation de NF-κB ainsi que son rôle dans les cellules myeloides infectées par le VIH. Des expériences menées dans les cellules U937 et PLB-985 ainsi que leurs lignées correspondantes infectées par le VIH, c'est-à-dire U9-IIIB et PLB-IIIB, ont révélé que NF-κB est constitutivement lié à l'ADN dans les cellules infectées par le VIH, un phénomène causé par une régulation altérée des kinases situées en amont de son activation. La kinase dépendante de l'ARN double brin (PKR) et la kinase d'IκB (IKK) sont constitutivement activées dans les cellules infectées, ce qui mène à la phosphorylation et à la dégradation des protéines inhibitrices IκB. Suite à la dégradation d'IκBα et IκBβ, NF-κB est libéré, permettant ainsi sa translocation du cytoplasme au noyau et l'activation de gènes cibles, incluant les sous-unités NF-κB p105/p50, p100/p52 et c-Rel. La quantité de ces sous-unités NF-κB est augmentée dans les cellules infectées par le VIH U9-IIIB et PLB-IIIB, ce qui peut contribuer à l'activation constitutive de NF-κB dans ces cellules. De plus, IκBβ est capable d'entrer dans le noyau et et de former un complexe ternaire en se liant aux protéines NF-κB associées à l'ADN. IκBβ maintient ainsi l'activité transcriptionelle de NF-κB en empêchant IκBα de le dissocier de l'ADN. La dégradation de IκBα et IκBβ mène donc à la libération et l'activation de NF-κB, et de plus IκBβ protège NF-κB lié à l'ADN d'en être dissocié par les protéines IκBα nouvellement produites.

Nous avons aussi examiné la fonction de l'activation constitutive des facteurs NF-κB et déterminé que ces derniers sont importants pour le maintient de la viabilité des cellules infectées par le VIH. L'apoptose induite par le TNFα est en relation inverse avec l'activation de NF-κB dans les cellules monocytaires et l'inhibition de NF-κB dans les cellules infectées par le VIH mène à la mort cellulaire par apoptose. Il semble donc que le

VIH induit l'activation des facteurs NF-κB dans les cellules infectées en modulant l'activité des kinases situées en amont de leur activation, ce qui empêche les cellules infectées de mourir par apoptose. Ces résultats ont d'importantes implications dans le traitement de l'infection par le VIH.

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Preface

In accordance with the guidelines for thesis preparation, I have chosen to present the results of my research in classical form. A General Introduction is presented in Chapter I, followed by detailed Materials and Methods in Chapter II. Chapters III, IV and V outline the results of my experimental work and appear in the following published manuscripts:

1. DeLuca, C., Roulston, A., Koromilas, A., Wainberg, M.A. and Hiscott J. 1996 Chronic human immunodeficiency virus type 1 infection of myeloid cells disrupts the autoregulatory control of the NF-κB/IκB pathway. *J. Virol.* **70:** 5183-5193.

2. Hiscott, J., Beauparlant, P., Crepieux, P., DeLuca, C., Kwon, H., Lin, R. and Petropoulos, L. 1997 Cellular and viral protein interactions regulating IκBα activity during human retrovirus infection. *J. Leukoc. Biol.* **62**: 82-92.

3. Kwon, H., Pelletier, N., DeLuca, C., Genin, P., Lin, R., Wainberg, M.A. and Hiscott, J. 1998 Inducible expression of IκBα repressor mutants interferes with NF-κB activity and HIV-1 replication in Jurκat T cells. *J. Biol. Chem.* **273**: 7431-7444.

4. DeLuca, C., Kwon, H., Pelletier, N. Wainberg, M.A. and Hiscott, J. 1998 NF-κB protects HIV-1 infected cells from apoptosis. *Virol.* **244**: 27-38.

5. DeLuca, C., Petropoulos, L., Zmeureanu, D. and Hiscott, J. 1999 Nuclear IκBβ maintains persistent NF-κB activation in HIV-1 infected myeloid cells. *J. Biol. Chem.* **274**: 13010-13016.

Chapter VI is a General Discussion and summary of the results presented in Chapters III through V. References for all chapters appear in Chapter VII.

The candidate was responsible for all the work presented in this thesis but would like to acknowledge the contribution of several experiments carried out by colleagues in the Hiscott laboratory: Anne Roulston for experiments presented in Figures 11 and 12. Dr. Antonis Koromilas for technical advice on the PKR kinase assays, Lousia Petropoulos and Christophe Heylbroek for help with the kinase assays in Figure 27, Dana Zmeureanu for technical assistance with Figures 22 and 23 and Hakju Kwon for generating the Jurkat Neo, Jurkat 2N and Jurkat 2NΔ4 cell lines used in experiments presented in Figures 34, 35 and Table 4.

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Table of Contents

List of Figures

Chapter IV

Chapter V

Chapter VI

List of Tables

Abbreviations

ADC	AIDS dementia complex
AIDS	Acquired immunodeficiency syndrome
AIF	Apoptosis inducing factor
ARM	Arginine rich RNA binding motif
ATP	Adenosine triphosphate
AZT	3'-Azido-2',3'-dideoxythymidine
BH	Bcl-2 homology domain
CA	Capsid HIV protein
CAD	Caspase activated deoxyribonuclease
CARD	Caspase recruitment domain
CAT	Choramphenicol transferase
CD4	Cluster determination antigen 4
CD8	Cluster determination antigen 8
CKII	Casein kinase II
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
CTD	C-terminal domain (Pol II)
ddC	2',3'-Dideoxycytidine , Zalcitabine
ddI	2',3'-Dideoxyinosine, Didanosine
d4T	2',3'-Didehydro-3'-deoxythymidine, Stavudine
DED	Death effector domain

DFF	DNA fragmentation factor
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
EBV	Epstein-Barr virus
eIF2α	Eukaryotic initiation factor 2α
ELAM-1	Endothelial leukocyte adhesion molecule-1
FADD	Fas associated death domain protein
FDC	Follicular dendritic cell
FLICE	Fas like ICE
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte/macrophage colony stimulating factor
HAART	Highly active anti-retroviral therapy
HBV	Hepatitis B virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HMG	High mobility group
HSV-1	Herpes simplex virus-1
HTLV-1	Human T cell leukemia virus-1
IAP	Inhibitor of apoptosis protein
ICAD	Inhibitor of CAD
ICAM-1	Intracellular adhesion molecule-1
ICE	Interleukin 1 converting enzyme

IFN	Interferon
IκB	Inhibitory kappa B protein
IL	Interleukin
IRF	Interferon response factor
LBP	Leader binding protein
LTR	Long terminal repeat
MA	Matrix HIV protein
MGSA	Melanoma growth stimulating activity
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
mRNA	Messenger RNA
NC	Nucleocapsid HIV protein
NEF	Negative factor HIV protein
NES	Nuclear export signal
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa B transcription factor
NLS	Nuclear localization signal
NRTI	Nucleoside reverse transcriptase inhibitor
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRE	Negative regulatory element
NSI	Non synticia inducing
NU	Neutralizing units

PAK	p21 activated kinase
PBMC	Peripheral blood mononuclear cells
PEST	Proline, glutamic acid, serine, threonine rich domain
PKR	dsRNA dependent protein kinase
PMA	phorbol myristate acetate
Pol II	RNA polymerase II
RHD	Rel homology domain
RNA	Ribonucleic acid
RNase H	Ribonuclease H
RRE	Rev response element
RT	Reverse transcriptase
SCID	Severe combined immunodeficiency
SDF-1	Stromal derived factor-1
SI	Synticia inducing
SU	Surface HIV protein
SV40	Simian virus 40
TAP1	Transporter protein
TAR	Tat responsive element
3TC	2',3'-dideoxy-3'-thiacytidine, Lamivudine
TM	Transmembrane HIV protein
TNF	Tumor necrosis factor
TNFR	TNF receptor
TRADD	TNFR associated death domain
TRAF	TNFR associated factor

TRAK	TNFR associated kinase
UBP	Upstream binding protein
VCAM-1	Vascular cell adhesion molecule-1
Vif	Virion infectivity factor protein
Vpr	Virion protein R
Vpx	Virion protein X

CHAPTER I

GENERAL INTRODUCTION

1.0 PATHOGENESIS OF HIV-1 INFECTION

Human immunodeficiency virus (HIV) is the causative agent of AIDS. AIDS or acquired immunodeficiency syndrome, has often been referred to as the modern day plague. The disease has had devastating effects on individuals and societies worldwide. Its physical destruction is often combined with social stigmatization, making AIDS truly a 20th century tragedy.

The disease caused by HIV was first identified in 1981, as a syndrome in young homosexual men and later in other populations including intravenous drug users, hemophiliacs and blood transfusion recipients. Soon after, a retrovirus was isolated from patient samples and named human immunodeficiency virus type 1 (HIV-1). A biologically distinct second type of HIV, HIV-2 was isolated in West Africa and shown to be similar to simian immunodeficiency virus, a retrovirus that induces an AIDS like syndrome in macaques. This set the stage for a flurry of basic and clinical research that has shed considerable light on the pathogenesis and treatment of HIV infection.

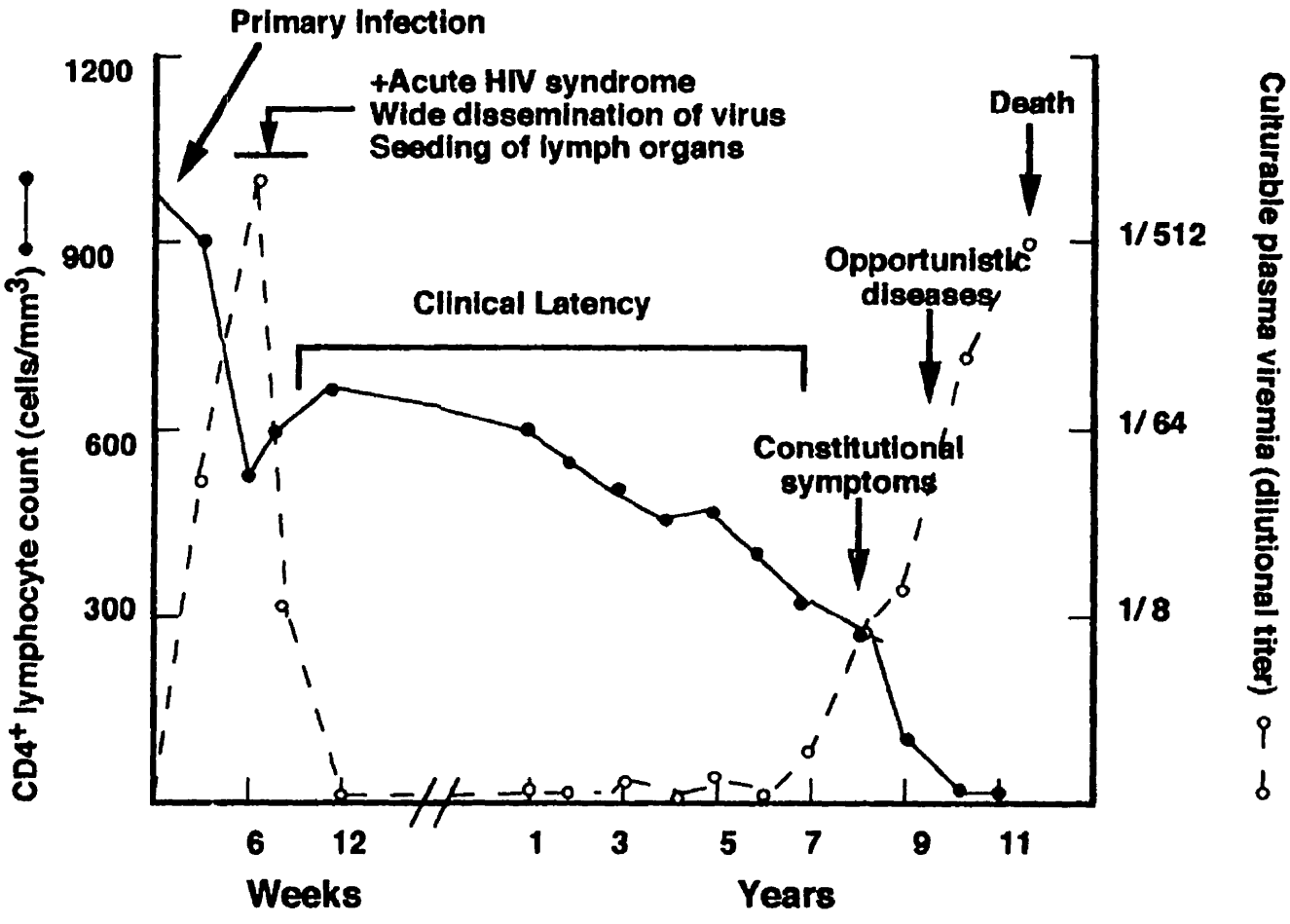
The scientific community has quickly responded to the challenge of understanding and combating this complicated retrovirus. Much has been learned about the mechanism of HIV immune destruction, the complicated host and viral interplay involved, and about therapies of medically managing people infected with HIV. Although much is known, the presently available therapeutic interventions only delay disease progression. Further investigations aimed at understanding the factors which influence gene expression and replication will form the basis of future, more effective antiretroviral strategies.

1.1 The Course of HIV-1 Infection - Stage 1

The development of AIDS is the result of a progressive infection with HIV, whose course and outcome are affected by interactions between viral and host cellular factors (171,368). Sexual transmission of HIV likely involves the initial infection of dendritic cells and cells of the monocyte lineage. These cells are permissive for HIV replication and transmit the virus to other susceptible targets such as $CD4^+$ T cells.

The progression of HIV infection, can be divided into four stages (refer to Figure 1). Initial infection is often followed by a short symptomatic period referred to as acute primary infection where infected individuals often experience flu-like symptoms, similar to mononucleosis (21). This stage is characterized by high viral replication, where virus can readily be isolated from blood (106), and massive $CD4^+$ T cell death. Virus dissemination occurs and lymph nodes become havens for persistent HIV replication. They are believed to be the primary anatomical site for the initial establishment of infection. The immune system responds with massive clonal expansion of $CD8^+$ HIV specific cytotoxic T lymphocytes (CTLs) and the eventual appearance of a humoral response. Evidence suggests that the immune system is already severely impaired during primary infection; subsequent to the expansion of $CD8^+$ CTLs, both HIV- specific $CD4^+$ T cell clones and HIV specific cytotoxic $CD8^+$ T cell clones undergo rapid deletion (171).

Figure 1. Clinical stages of HIV-1 infection. HIV-1 infection is characterized by four stages: CD4+ T cell numbers drop dramatically and levels of virus in the periphery peak in the first stage; plasma viremia drops to almost undetectable levels and CD4+ T cells initially rebound and begin to slowly decrease with time in the second stage; the onset of symptomatic HIV infection characterizes the third stage; virus replication dramatically increases and virus can be isolated from as many as 1% of CD4+ T cells in the fourth stage. Death generally follows within 1 to 2 years. Redrawn from (136).


Primary infection
+Acute HIV syndrome
Wide dissemination of virus
Seeding of lymph organs
Death
Opportunistic diseases
Clinical Latency
Constitutional symptoms
1200
900
600
300
1/512
1/64
1/8
CD4+ lymphocyte count (cells/mm3)
Culturable plasma viremia (dilutional titer)
6
12
1
3
5
7
9
11
Weeks
Years

1.2 The Course of HIV-1 - Stage 2

The second stage is referred to as the clinical latency period which can last from 2-15 years and in which individuals are generally asymptomatic. The number of $CD4^+$ cells rebound initially and then experience a slow decrease. The widespread destruction of T cells continues but is paralleled by a heightened rate of T cell production, stabilizing immune cell function. Viral replication is markedly reduced in the peripheral blood stream (194) and is followed by the appearance of neutralizing antibodies (171). Humoral and cell mediated responses although vigorous, are generally unable to contain the infection and HIV replication persists in the lymph nodes (130,329,330).

1.3 The Course of HIV-1 Infection - The Final Stages

The development of symptomatic HIV infection characterizes the third stage. Individuals may show signs of lymphadenopathy, diarrhea, weight loss, recurrent candidal infections and peripheral neuropathy. The onset of any of these symptoms is an indicator that the disease is likely to progress to AIDS within the next two to three years (175).

Full blown AIDS is the final stage of the disease. HIV replication dramatically increases and can be isolated from as many 1% of $CD4^+$ T cells (386,388). HIV isolates obtained late in disease are T cell tropic, and able to replicate in T cells and T cell lines but not in primary monocytes. These viruses are characterized by rapid replication and syncitia formation and signal the demise of the immune system (140,422).

2.0 HIV-1 REPLICATION

2.1 HIV-1 Structure

The structure of the HIV-1 virion has been elucidated by high resolution electron microscopy. The virion has an icosahedral structure (162) encased in a lipid bilayer containing 72 external spikes composed of the two major viral envelope proteins, gp120 and gp41 (see Figure 2 for schematic). Several host proteins can also be found in the lipid bilayer, including class 1 and class 2 major histocompatability (MHC) proteins which are acquired during virus budding. The HIV-1 core contains four polypeptides, p24, p17, p9 and p7, which are processed from a 53-kDa Gag precursor and together form the nucleocapsid. p24 is the major component of the capsid core structure, p17 associates with the inner surface of the lipid bilayer and p7 directly binds the RNA genome through a zinc finger motif and, together with p9, forms the nucleoid core. The core also contains two copies of the single stranded RNA genome, the viral enzymes reverse transcriptase, RNase H, integrase and protease, and minimal amounts of regulatory proteins such as Nef, Vpr and Vif (102,434).

2.2 HIV Genome

HIV-1 is a member of the Lentiviridae subfamily of retroviruses and comparison of the genomic organization of primate lentiviruses, reveals that the Gag, Pol, Env, Vif, Tat, Rev and Nef are highly conserved (see Figure 3 for schematic of HIV-1 genome). HIV-1 has two additional genes, *vpr* and *vpu* while HIV-2 and some strains of SIV, lack the *vpu* gene but contain a gene upstream of *vpr*, called *vpx* (434). The Tat and Rev regulatory proteins have coding sequences that overlap each other. The genes of HIV-1 are contained in a 9 kB RNA genome, flanked by long terminal repeat (LTR) sequences which provide important regulatory information (discussed below).

Figure 2. Schematic representation of HIV-1 virion. The virus is an icosahedral structure encapsulated in a lipid bilayer containing 72 external spikes composed of gp120 and gp41. The HIV-1 core contains capsid, matrix, nucleocapsid and p7 viral proteins as well as two copies of the single stranded RNA genome, the viral enzymes RT, integrase and protease and minimal amounts of the regulatory proteins Nef, Vpr and Vif.

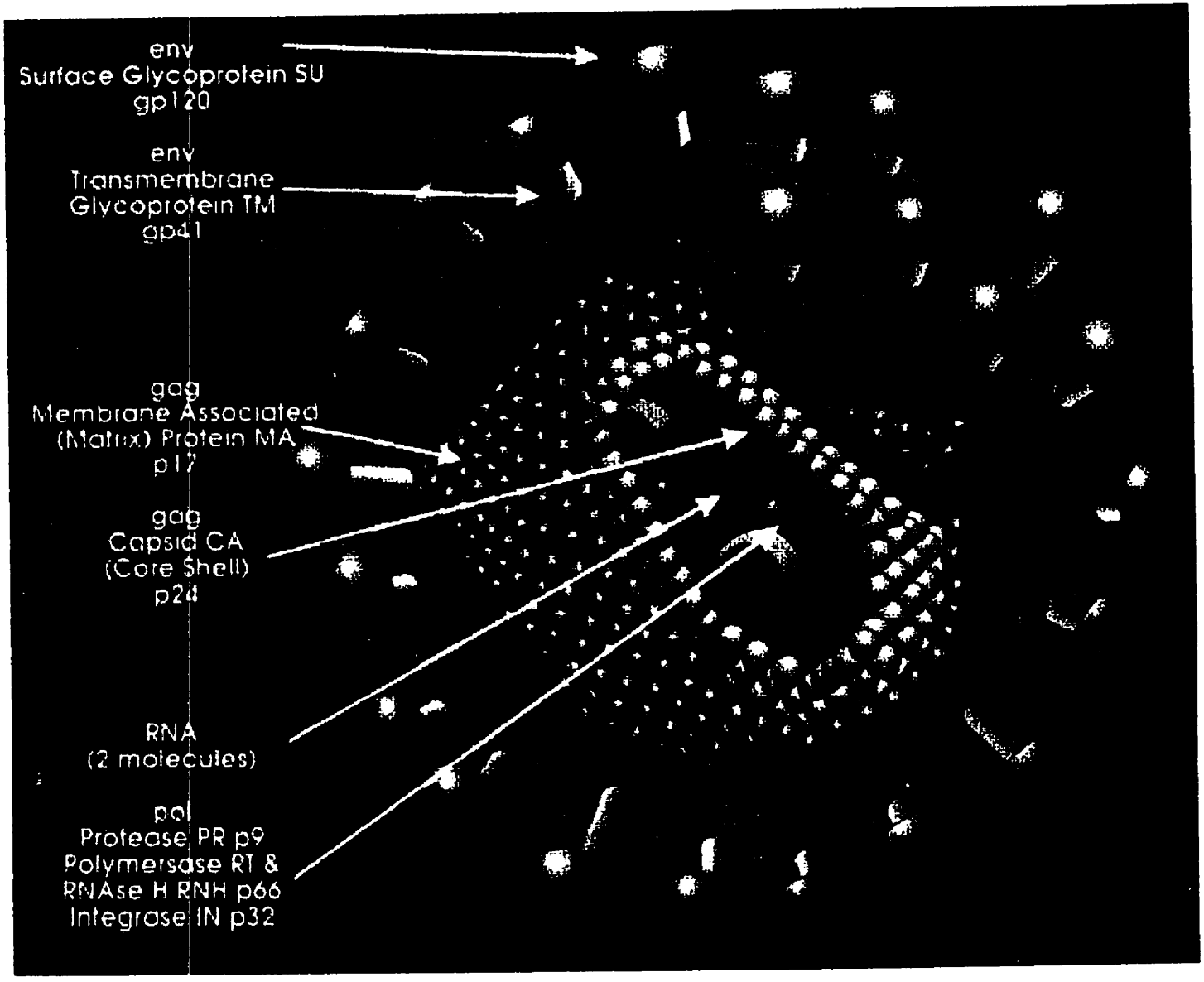
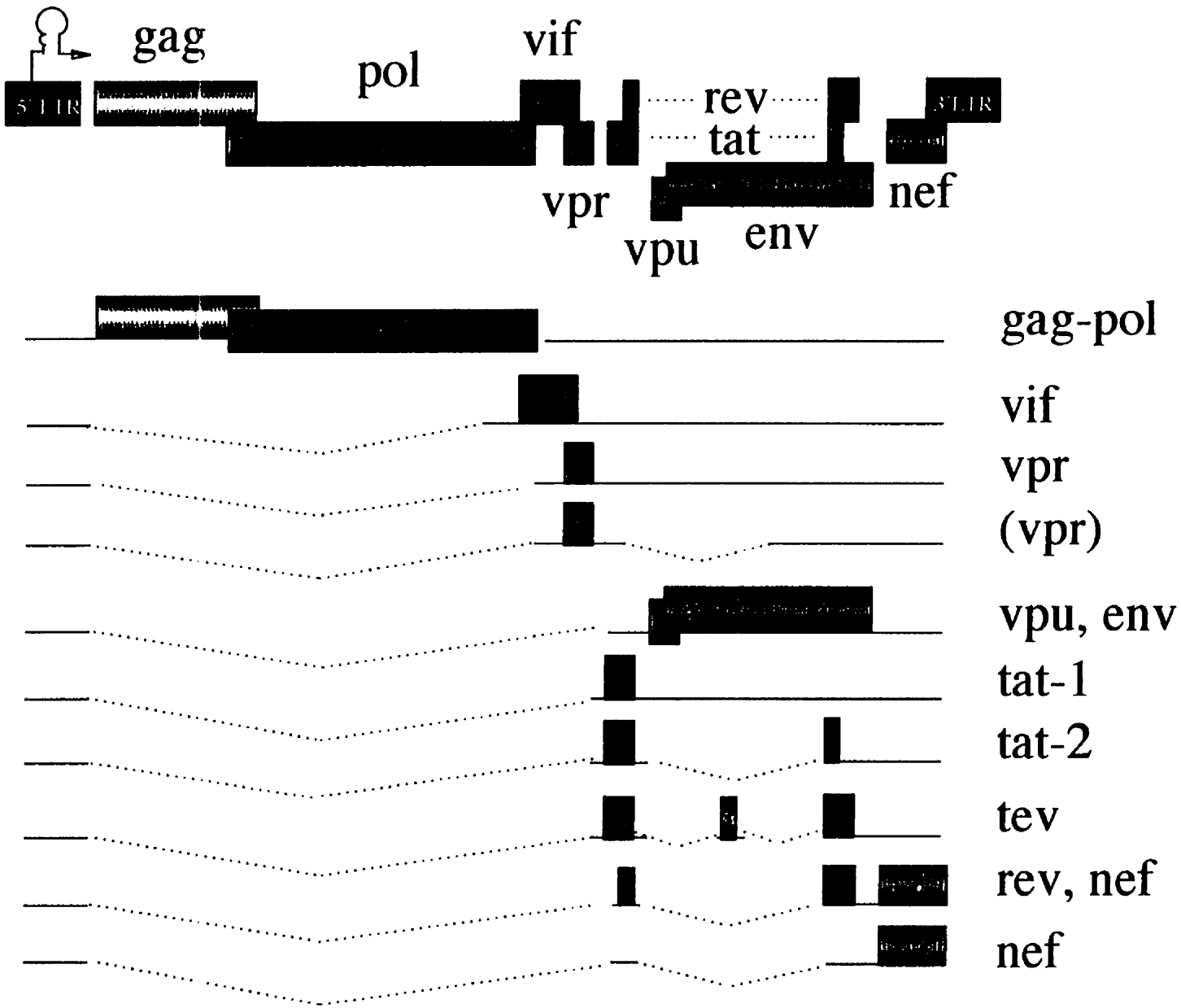

env
Surface Glycoprotein SU
gp120
env
Transmembrane
Glycoprotein TM
gp41
gag
Membrane Associated
(Matrix) Protein MA
p17
gag
Capsid CA
(Core Shell)
p24
RNA
(2 molecules)
pol
Protease PR p9
Polymersase RT &
RNAse H RNH p66
Integrase IN p32

Figure 3. Schematic representation of the HIV-1 genome and the splicing pattern of HIV-1 transcripts. The gag and gag-pol mRNA's are unspliced, mRNA's for the early genes are doubly spliced, and mRNA's for the late genes are singly spliced. Redrawn from (153).


gag
pol
vif
rev
tat
vpr
nef
vpu
env
gag-pol
vif
vpr
(vpr)
vpu, env
tat-1
tat-2
tev
rev, nef
nef

2.3 HIV-1 Viral Gene Products

2.3.1 *Structural proteins*

The HIV-1 genome codes for at least 9 gene products. A cell first encounters the envelope proteins gp120 (also called SU for surface protein) and gp41 (also called TM for transmembrane protein), which together bind target receptors, and fuse with the host plasma membrane. As their name implies, the SU subunit comprises the extracellular portion of the envelope dimer, and TM spans the lipid membrane, anchoring the complex to the virion surface. These envelope proteins are first synthesized as a precursor protein, gp160, which is cleaved by a cellular protease, and the two subunits noncovalently interact. Newly synthesized gp160 can interact with and retain CD4, and the associated cytoplasmic tyrosine kinase, p56lck, in the endoplasmic reticulum. This may be important for preventing cell aggregation and may contribute to T cell dysfunction (97,98).

As previously mentioned, the precursor Gag protein is cleaved by the HIV-1 protease into p17, p24, p9 and p6 proteins. p17 or matrix (MA) protein lines the inner surface of the virion and interacts with the lipid bilayer via its myristic acid moiety (153). p17 functions to target the Gag precursor to the membrane and is important for virion stability and maturation. It is also thought to play a role in viral uncoating and transport of the pre-integration complex (59,60), although this function has recently been disputed (151). p24 or capsid protein (CA) is the major capsid protein forming the viral core and is important for viral uncoating, potentially via its interaction with the cellular chaperone protein cyclophilin A (273). p9 is the nucleocapsid protein (NC) which binds tightly to the viral RNAs, forming a link between the two viral genomes. Finally, the p7 protein, which comprises the C-terminal 51 amino acids of Gag, is important for incorporation of Vpr during viral assembly (4).

2.3.2 *HIV-1 enzymes*

The HIV-1 Pol protein is translated from the same transcript as the Gag precursor by a novel ribosomal frame shifting mechanism resulting in a -1 nucleotide frameshift and is referred to as Gag/Pol (207). The frame shifting mechanism is inefficient and approximately 20 times more Gag protein than Pol is produced. The Pol protein is autoproteolytically cleaved after virion budding by the HIV-1 encoded protease. In addition to protease (p10), Pol cleavage produces reverse transcriptase (p66/p51) and integrase (p32). The protease also processes Gag precursor protein. Reverse transcriptase is an RNA-dependent DNA polymerase which translates viral genomic RNA into complementary DNA. This enzyme also has ribonuclease H (RNase H) activity, which is required for second strand DNA synthesis. The integrase enzyme catalyzes the insertion of proviral DNA into the host genome, making HIV-1 a permanent resident of the cell.

2.3.3 *Tat*

HIV-1 also codes for several auxiliary proteins which are important regulators of virus replication and disease progression. Tat, arguably the most studied HIV auxiliary protein, is a potent transcriptional activator of the HIV-1 LTR and is essential for viral replication (102). Tat accomplishes this function by interacting with an RNA stem loop structure, termed the Tat response element (TAR), found immediately upstream of the transcription start site. The 59 base pair stem loop structure has two critical sequence elements: a hexanucleotide loop and a three nucleotide U rich bulge located four nucleotides upstream of the loop. Both the bulge and the loop are critical for Tat transactivating function *in vivo*. Tat protein can be synthesized as a one exon or two exon protein, both containing the two functional domains. Tat has a cofactor binding domain in its extreme N-terminus and an arginine rich RNA-binding motif (ARM) immediately adjacent. The ARM domain also serves as a nuclear localization signal (NLS), permitting it to enter the nucleus (102). Tat is thought to increase both transcript processivity of

initiated RNA polymerase II (Pol II) molecules and increase LTR transcriptional initiation (247,473), although this latter function is disputed (101,141). Recently, the mechanism of Tat mediated elongation of HIV-1 transcripts has been elucidated. Tat interacts with a protein termed cyclin T, which in turn, specifically interacts with the kinase, CDK9 (446,473,488). CDK9 can phosphorylate the C-terminal domain (CTD) of Pol II, leading to increased transcript processivity. Hence, Tat functions to increase the transcription of full transcripts by recruiting CDK9 to the transcription elongation complex.

Tat has also been suggested to function as a cytokine, activating cells in some contexts (12,326,456), inducing apoptosis in others (40,257,284). Tat can induce TNFα production, activating NF-κB and perpetuating TAR independent HIV LTR gene transcription (471). Tat can also co-operate with NF−κB and other transcription factors to induce synergistic activation of the HIV LTR (223,311), further augmenting viral gene expression. Neural toxicity causing ADC has also been linked to Tat expression through its ability to activate TNFα expression in macrophages and astrocytes (71,243).

2.3.4 *Rev*

Rev is also essential for virus replication (reviewed in 102,153) and functions as a sequence specific RNA export protein. Since HIV-1 contains only one promoter element, the HIV LTR, it encodes only one genome length primary transcript. Expression of the nine HIV-1 viral genes requires that an unspliced variant, singly spliced transcripts as well as many multiply and fully spliced mRNA's are expressed in the cytoplasm (Figure 3). Rev functions to permit cytoplasmic shuttling of unspliced and partially spliced mRNAs by inhibiting the host's splicing machinery and overriding the cell's attempt to retain unspliced mRNAs in the nucleus. In the absence of Rev, only fully spliced transcripts of HIV are found in the cytoplasm, thereby preventing virus replication. Rev accomplishes this feat by interacting with an RNA target site named the Rev response element (RRE).

The RRE is a 234 nucleotide RNA stem-loop structure and Rev likely binds the RRE bulge region as a monomer (102). The initial binding of Rev serves to recruit additional Rev monomers to the RRE. Rev binding is mediated by the arginine rich N-terminal ARM domain which also serves as a nuclear localization sequence (NLS), giving Rev ready access to the nucleus. This region is flanked by residues involved in Rev multimerization and by a nuclear export sequence (NES) located further upstream. Rev carries HIV-1 mRNAs out of the nucleus by recruiting the nuclear export protein, Crm1 (102,149,314,453). Rev/Crm1 subsequently bind the nuclear pore export machinery and shuttles the Rev-RRE-RNA complex into the cytoplasm (490).

2.3.5 *Nef*

The largest and most highly expressed auxiliary protein is Nef (reviewed in 102). Nef was initially considered a *ne*gative *f*actor due to its modest effect in most tissue culture settings but was later shown to have several important functions. Nef induces down regulation of cell surface CD4 expression, possibly preventing CD4 from complexing with newly synthesized HIV-1 virions (161,279), ensuring progeny viruses are released, and can also down-regulate cell surface expression of MHC 1 proteins (251). Since MHC 1 proteins are required to present viral epitopes to CTLs, downmodulation of cell surface MHC 1 inhibits CTL mediated lysis of HIV-infected cells (93). In addition, Nef affects several signaling pathways and can alter the activation of lymphoid and non-lymphoid cells. Recent evidence suggests that Nef harbors a major determinant for HIV-1 associated pathology. Remarkably, Nef mutation in HIV-1 expressing transgenic mice, completely reverses the HIV disease like phenotype exhibited by mice expressing wild type HIV-1 (178). Transgenic mice carrying an HIV-1 genome mutated for all genes except Nef, displayed all the phenotypes of wild type HIV-1 transgenic mice suggesting all other genes were dispensable for disease development. Moreover, the level of Nef expression correlated with disease progression, implying a pivotal role for Nef in AIDS pathology.

2.3.6 *Vif, Vpr and Vpu*

Vif, or virion infectivity factor, is produced late in the HIV-1 lifecycle and functions to enhance the infectivity of HIV-1 virions (279,434). Vpr is also a late stage HIV-1 gene product and is packaged in virion progeny in quantities comparable to the Gag protein. Vpr mediates the import of HIV-1 pre-integration complexes into the nucleus (183), a function which is particularly important for growth arrested cells such as macrophages. Nuclear import is effected by an atypical NLS sequence in conjunction with NLS sequences found in matrix and integrase proteins (60,102,129). In addition, Vpr can induce G2 cell cycle arrest (129). Recent studies suggest that the HIV-1 LTR is more active in G2 arrested cells, and hence the Vpr induced block in G2 may serve to enhance LTR mediated gene transcription (5,166). Unlike the other auxiliary proteins which are conserved in all primate immunodeficiency viruses, Vpu is unique to HIV-1 and the closely related chimpanzee SIV (SIV_{cpz}) isolates. Vpu is important for selectively degrading CD4 in the cell endoplasmic reticulum (459), as well as enhancing HIV-1 virion release (230,279). Vpu facilitates both the targeting of budding virions to the plasma membrane (instead of intracytoplasmic membranes), and promotes their release from the cell surface (230). The mechanism employed remains to be completely elucidated, although the hydrophobic transmembrane tail of Vpu is known to be required for this activity.

2.4 HIV-1 Receptors and Cell Tropism

HIV-1 requires two receptors to enter susceptible cells: cluster determinant 4 (CD4) receptor and a chemokine receptor. CD4 receptor has long been recognized as the primary receptor for HIV-1. The identification of chemokine receptors as HIV-1 coreceptors is a much more recent finding (reviewed in 122,378). Chemokine receptors are a family of seven transmembrane G-protein coupled receptors involved in inflammation and

hematopoiesis (350). Two main chemokine receptors are used by HIV-1 and determine the tropism of the virus. CCR5 is the major chemokine receptor used by macrophage or M-tropic (also R5) strains of HIV-1 (115,124). M-tropic viruses are able to replicate in primary macrophages and T cells but do not infect transformed T cell lines. They replicate more slowly and are less virulent than their T-tropic relatives. T cell tropic or T-tropic (also X4) viruses employ the CXCR4 chemokine receptor to gain entry into host cells (139). These viruses usually appear late in disease, are associated with increased AIDS pathology, and induce syncitia formation. Other chemokine receptors have also been shown to permit infection by certain HIV-1 strains, including CCR2 and CCR3 (82,123,124). The role of these receptors is less clear however, since CCR5 and CXCR4 remain the receptors used by all known strains of HIV-1. Several HIV-1 isolates can use both CCR5 and CXCR4 and are referred to as dual tropic or R5X4 viruses (123,124).

Interestingly, some cases of CD4 independent HIV-1 and HIV-2 infection have recently been reported: the viruses required only CXCR4 (132,357). Several strains of SIV are also known to use CCR5 in the absence of CD4 (127). Although, CD4 is important for concentrating HIV-1 at the cell surface and is generally necessary for cell entry, it may, given that no cases of chemokine receptor independent viral entry have been reported, be more correct to refer to chemokine receptors as the primary receptor and CD4 as the coreceptor.

2.5 HIV-1 Lifecycle

2.5.1 *Early events*

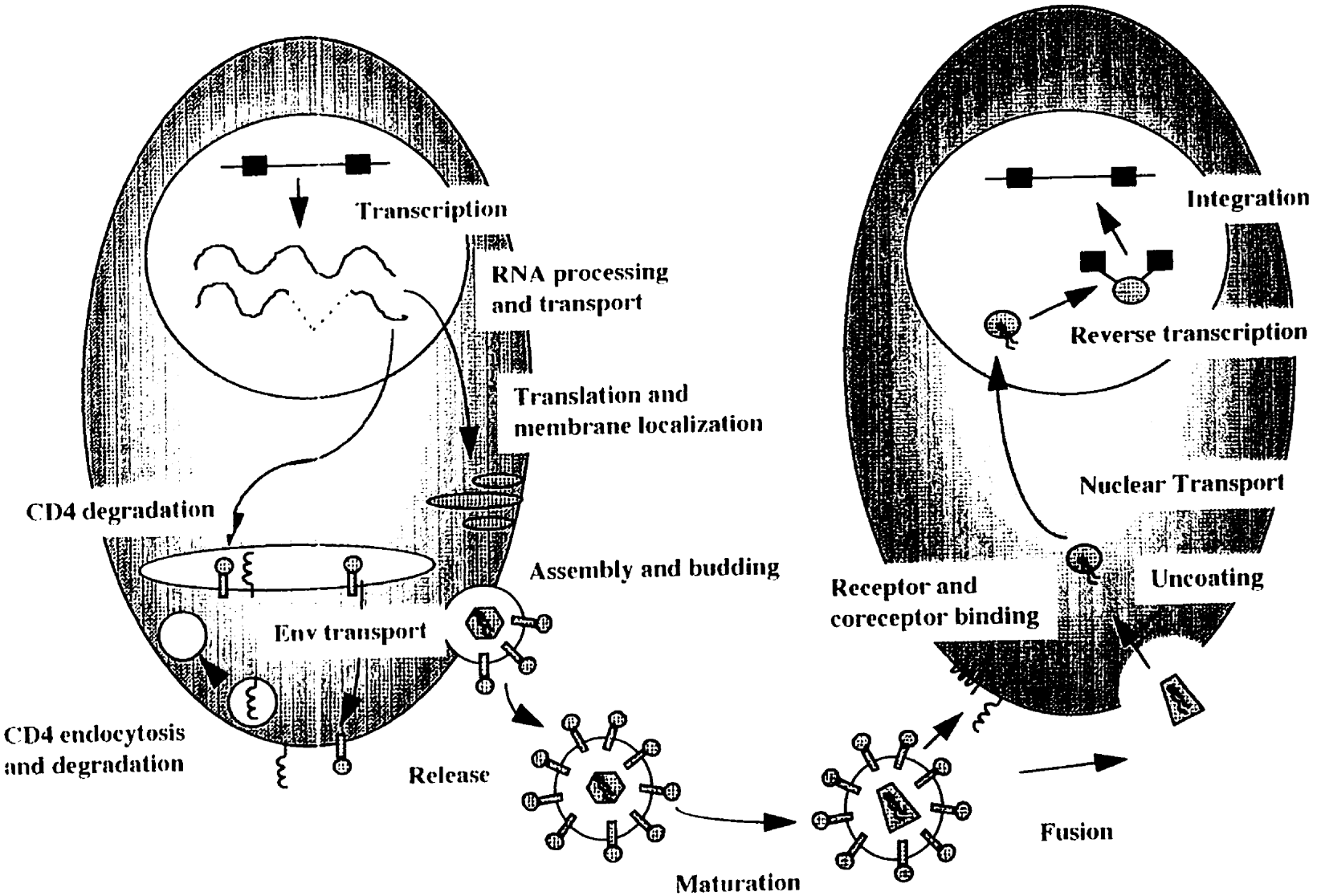
HIV-1 infects $CD4^+$ cells including $CD4^+$ T cells, cells of the monocyte lineage and dendritic cells (63,64,231,421). Dendritic cells and cells of the monocyte lineage are likely the first cells infected by HIV (63,171,341) and have been implicated as perpetrators of

viral spread. HIV-1 binds the CD4 receptor on susceptible cells via its highly glycosylated surface protein gp120. A conformational change is thought to take place which allows gp120 to bind its appropriate coreceptor (269). The transmembrane gp41 protein is then able to interact with its target cell membrane, causing fusion of the viral envelope with the host cell membrane. The viral particle is subsequently internalized and partially uncoated (439). Viral replication begins with the generation of a first-strand DNA copy of the viral RNA genome and is mediated by the HIV-1 encoded RT. RNase H partially degrades the original RNA template allowing the second strand of DNA to be synthesized. Replication of the RNA genome involves two strand transfer reactions where reverse transcriptase can jump from one strand of the RNA genome to the other. A double stranded DNA replica of the viral genome is made that contains an LTR at each end of the DNA. The proviral DNA duplex is then inserted into the host genome by viral integrase and synthesis of viral gene products is ready to begin. Figure 4 illustrates the steps involved in HIV-1 replication (reviewed in 201).

2.5.2 *Late events*

HIV can establish a productive or latent infection (171,286). Analysis of peripheral blood $CD4^+$ T cells reveals that only 10% of infected cells actually produce HIV-1 RNA (388). Productive infection begins with the expression of HIV regulatory genes (refer to Figure 4) (153). Host transcription factors bind the HIV-1 LTR and induce a low but important level of HIV-1 gene expression. Only the fully spliced 2 kB transcript is efficiently

Figure 4. Model of HIV-1 replication. mRNAs are transcribed from the integrated HIV-1 genome. mRNAs are spliced and transported out in the cytosol. The early regulatory proteins are transcribed. Newly synthesized Rev protein permits the release of unspliced mRNAs and synthesis of structural proteins which localize to the membrane. HIV-1 proteins Nef and Env induce CD4 endocytosis and degradation. The HIV-1 virus is assembled at the membrane and buds, encapsulated in a plasma membrane envelope. Virus is released and cleavage of precursor proteins by the viral protease produces the mature virus. HIV-1 fuses with CD4+ cells expressing chemokine coreceptors. The virus is internalized and uncoated. The virus core is transported to the nucleus where the RT enzyme produces a DNA copy of the RNA genome that is inserted into the host's DNA by a reaction catalyzed by the viral enzyme, integrase. Adapted from (153).


Transcription
RNA processing
and transport
Translation and
membrane localization
CD4 degradation
Assembly and budding
Env transport
CD4 endocytosis
and degradation
Release
Maturation
Integration
Reverse transcription
Nuclear Transport
Uncoating
Receptor and
coreceptor binding
Fusion

produced at this stage (226) and codes for the regulatory proteins Tat, Rev and Nef. These proteins permit synthesis and cytoplasmic transport of longer unspliced and partially spliced transcripts and enhance HIV replication. The unspliced gag/pol mRNA and the singly spliced env mRNAs produce the viral enzymatic and structural proteins respectively; the former transcript also functioning as the viral genome which is packaged in viral particles. Assembly of progeny virions takes place at the plasma membrane. Newly synthesized HIV envelope proteins are inserted into the host cell membrane while the Gag polyprotein assembles with the Gag/Pol precursor, the accessory proteins Vpu and Vpr, and the RNA genome, and transports them to the cell surface. Once assembled, these cores bud through the plasma membrane, acquiring a lipid membrane seeded with HIV-1 envelope proteins. After budding, the HIV-1 protease processes capsid and viral enzyme proteins, leading to the maturation of infectious viral particles (Figure 4)

3.0 MOLECULAR BIOLOGY OF HIV-1 GENE EXPRESSION

HIV-1 gene expression involves a complex interplay of host and viral proteins. The virus has usurped many host cellular gene regulatory strategies and devised unique solutions to combat regulatory problems, adapting its own proteins to complement host protein functions. By these mechanisms the virus has maximized its potential for replicative success. This section will focus on host transcription factors and their importance in HIV-1 gene expression.

3.1 Promoter Elements Regulating HIV-1 Gene Expression

3.1.1 *Cellular regulators of basal transcription*

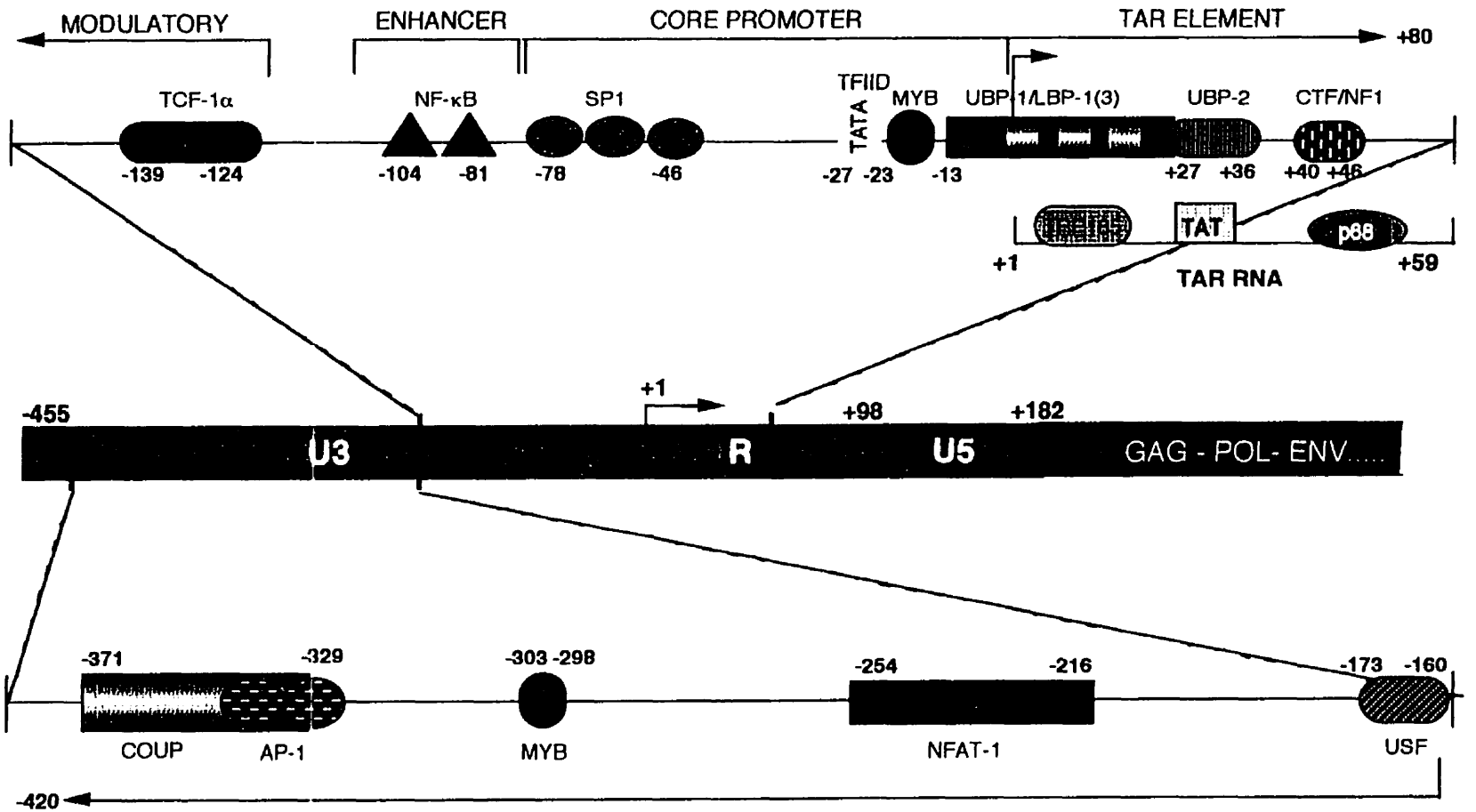
Numerous promoter elements in the U3 and R regions of the LTR have been described (refer to Figure 5). Both positive and negative elements have been identified as well as,

regions regulating basal and induced gene transcription (reviewed in 51). Basal HIV transcription has been detected in the absence of Tat or other inducible cell factors and depends on elements proximal to the transcription start site. HIV-1 contains a TATA box which is similar to those found in most eukaryotic promoters. Although important, mutation of the TATA box reveals that other factors are important for basal transcription (reviewed in 51). Leader binding protein (LBP-1) or upstream binding protein (UBP-1) (212) has been shown to bind several sequences around the TATA box. Interaction with its high affinity binding site plays an important role in supporting HIV basal transcription (212) although interaction with its low affinity site is reportedly repressive (219). Three binding sites for the mammalian cellular transcription factor, Sp1 have been identified 5' to the HIV TATA box (211). Sp1 is a strong transcriptional activator, containing three zinc finger motifs important for DNA binding and two glutamine rich transcription activation domains. Sp1 is important for HIV-1 basal (180) as well as Tat induced transcription (51,180).

3.1.2 *Cellular regulators of induced HIV-1 transcription*

Induced HIV transcription is principally regulated by the NF–κB family of transcription factors. Mitogen and phorbol ester stimulation of HIV-1 transcription is mediated by two identical NF–κB sites (311), located in the HIV-1 enhancer (-80 to -105 bp), just 5' of the Sp1 sites (Figure 5). HIV-2 has only one NF–κB site in its LTR. Through the duplication of the NF–κB site, HIV-1 can exploit the synergistic effect of the κB sites on transcriptional activation (344). NF–κB can be induced by a number of physiological activators such as antigen, viruses, UV light, H_2O_2 (generated by neutrophils during infection), inflammatory cytokines and bacterial toxins. The significance of these inducers as cofactors for AIDS progression has been postulated to be related to their ability to activate NF–κB (30).

Figure 5. Schematic representation of the regulatory elements in the HIV LTR. The viral LTR is divided into three regions designated U3, R and U5. The viral promoter is located in the U3 region and consists of three transcriptional domains; the core promoter, the enhancer and the modulatory domain. Transcription initiates at the U3/R border in the 5' LTR and the R region encodes the trans-activation response element, TAR. The R/U5 boundary in the 3' LTR defines the 3' end of all viral transcripts and is determined by the polyadenylation sequence in R.



MODULATORY/NEGATIVE REGULATORY ELEMENT

HIV-1 infection of monocytes can itself activate NF–κB (25) and may explain the persistent infection of these cells in tissue culture (152). The mechanism has not been clearly identified but may result from the production of TNFα and IL-1, creating an autocrine loop for NF–κB activation (347,349,372).

Studies have shown that Tat mediated amplification of HIV transcription of blood $CD4^+$ T cells requires functional κB sites (13) and Tat-TNF synergism is also largely due to NF–κB activation (44,52). In addition, activation of NF–κB strongly upregulates HIV gene expression in chronically infected cell lines (125,173), indicating an important role for NF–κB in the activation of latent viral infection. Although NF–κB strongly enhances HIV-1 viral replication, it is not absolutely required for virus growth (69). Activation by bacterial or viral co-infection however could activate latent proviral infections as well as greatly induce replication in productively infected cells, leading to massive viremia and cell destruction.

3.1.3 *Upstream host transcription factors*

Deletion of sequences upstream of position -155 leads to increased basal and induced LTR transcription and has hence been called the negative regulatory element (NRE) (Figure 5) (272). It includes the negative regulatory factor binding site which binds members of the helix-loop-helix family of proteins and may incorporate the NFAT-1 (nuclear factor of activated T cells) binding site (272), although evidence for the latter is contradictory (486). The region from -350 to 300 bp contains several transcription binding sites including potential sites for Ap1 (155), members of the steroid thyroid hormone receptor family (94), c-Myb (109), and NRT 1 and 2 (468), and also exerts a negative effect on HIV-1 transcription levels.

3.2 The NF–κB Family of Transcription Factors

The NF–κB family of transcription factors is involved in the transduction of immunological responses, cellular differentiation, and cell growth (reviewed 36,373,442). Gene knock out and other studies firmly establish a role for NF–κB in immunity and the inflammatory process but also indicate that NF–κB has other functions, including roles in liver development and disease processes. Table 1 lists the many genes known to be transactivated by NF-κB. Several properties make NF–κB an attractive transcription factor for viral appropriation: activation of NF–κB does not require new protein synthesis, NF–κB is a strong transcriptional activator and activation of NF–κB is rapid, taking place within minutes following exposure to a relevant inducer.

Several DNA binding family members exist. p50 and p52 are thought to be synthesized as the inactive precursors p105 (NF–κB1) and p100 (NF–κB2) respectively, and proteolytically cleaved to produce the mature DNA binding proteins. One report, however, suggests that p105 and p50 are synthesized separately from the same mRNA by a cotranslational regulation mechanism that depends on protein folding (263). RelA (p65), c-Rel and RelB are not synthesized as precursor proteins and contain C-terminal transactivation domains that resemble the one found in VP-16. All NF–κB proteins contain a Rel homology domain (RHD) in their N-terminus of approximately 300 amino acids, which mediates DNA binding, dimerization, and interaction with the inhibitory IκB proteins. Figure 6 schematically represents the functional domains of the NF–κB family members.

NF–κB is a dimer of protein subunits, homo- and heterodimer combinations allow NF–κB to selectively and differentially regulate gene expression (265). The classical NF–κB dimer is composed of RelA and p50 and preferentially binds the sequence 5'

Table 1. Genes regulated by the NF-κB family of transcription factors.

Class	Target Genes
Viruses	Human immunodefiency virus (HIV-1)
	Cytomegalovirus (CMV)
	Simian virus 40 (SV40)
Cytokines and hematopoietic growth factors	β-interferon
	Granulocyte/macrophage colony-stimulating factor (GM-CSF)
	Granulocyte colony-stimulating factor (G-CSF)
	Melanoma growth stimulating activity
	Interleukin 1β (IL-1β)
	Interleukin 2 (IL-2)
	Interleukin 6 (IL-6)
	Interleukin 8 (IL-8)
	Tumor necrosis factor α (TNF-α)
	Lymphotoxin β (TNF-β)
	Proenkaphalin
Cell adhesion molecules	Endothelial leukocyte adhesion molecule 1 ELAM-1)
	Vascular cell adhesion molecule 1 (VCAM-1)
	Intracellular adhesion molecule 1 (ICAM1)
Acute phase proteins	Angiotensinogen
	Serum amyloid A precursor
	Complement factor B
	Complement factor C4
	Urokinase-type plasminogen activator
Immunoreceptors	Immunoglobulin κ light chain (human)
	T cell receptor β chain
	T cell receptor α chain (human)
	Major histocompatability class II ($E\alpha^d$)
	β2-microglobulin
	Tissue-factor 1
Transcription factors and subunits	c-Rel
	NFκB1 (p105) and NFκB2 (p100)
	IκBα
	c-myc
	Interferon regulatory factor 1
	p53
Apoptotic regulators	Bcl-2
	c-IAP1 and 2
	TRAF-1 and -2
	Fas, FLICE
	TNF receptor 1 and 2

Reproduced from reference (30).

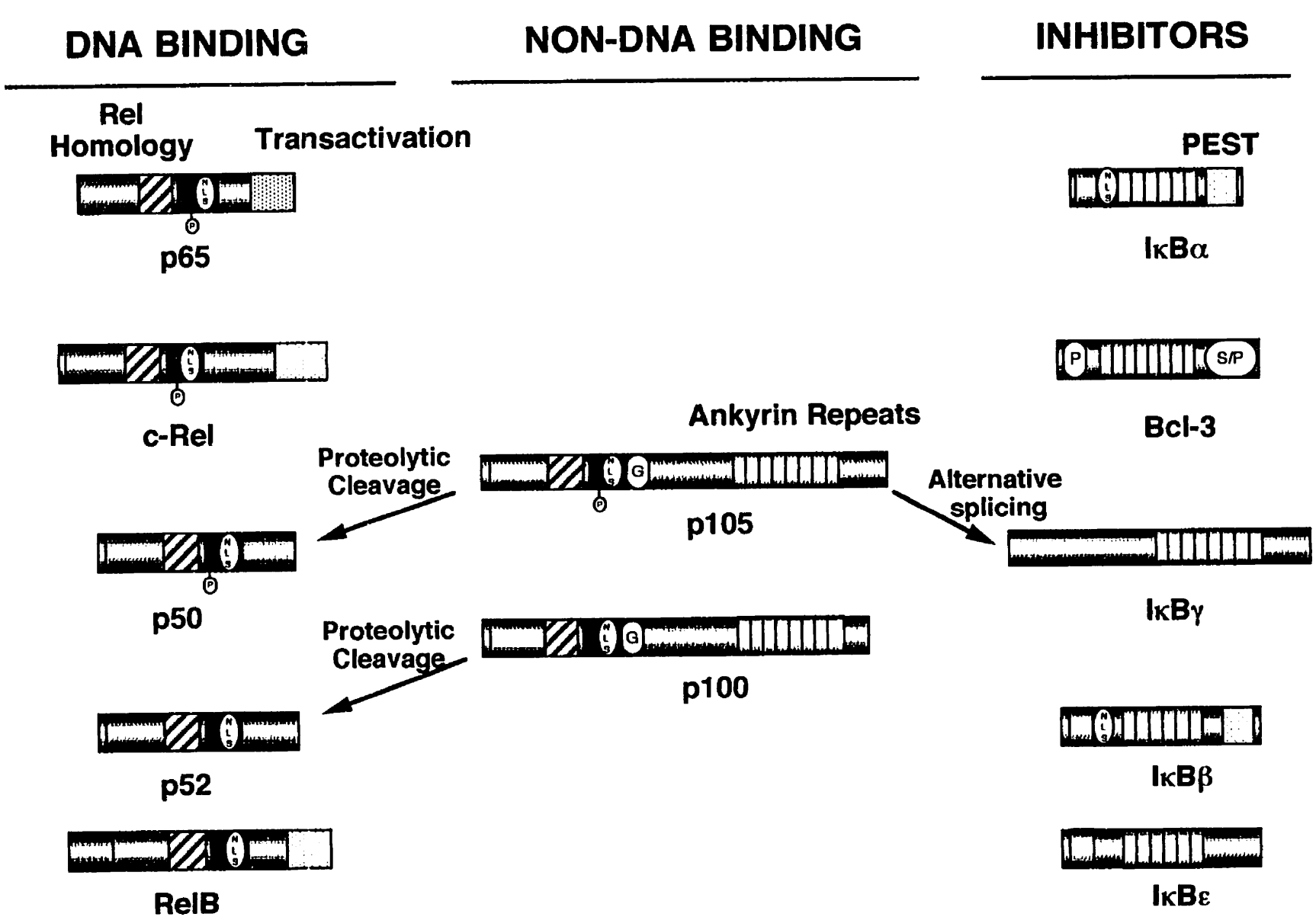
GGGRNNYYCC 3'(R = purine, Y = pyrimidine), containing a slightly different half site sequence. p50 homodimers bind more efficiently to palindromic motifs with identical sequences in their half sites while c-Rel/RelA dimers prefer the sequence 5' HGGARNYYCC 3'(where H = A, C or T). Activation potential varies greatly among the different NF-κB dimers with classical NF-κB often exhibiting the strongest transactivation potential. The function of p50 homodimers appears to depend on the context of the κB sequence. Some experiments have found that p50 homodimers have no effect on transcription (225), others have found a positive effect on gene transcription (56,304,306), while others yet suggest a negative role (312,346). Some evidence suggests that binding of the IκB protein, Bcl-3 to p50 homodimers, may confer transcriptional activation properties (157). Additional mechanisms allowing for differential gene regulation by NF−κB include cell type specificity, subcellular localization, regulation by different IκB members and differential activation (30,396).

Gene knockout studies have confirmed that NF−κB subunits are not functionally equivalent. Mice lacking p50/p105 develop normally but exhibit defects in B cell function (394). Mice deficient in RelB do not exhibit developmental defects but are profoundly impaired in immune function and hematopoiesis (62,448). Most dramatically, mice lacking the RelA gene die *in utero* due to massive apoptosis of cells in the liver (48). Embryo livers appeared normal at day 14 but underwent extensive apoptosis by day 16. This set the stage for the subsequent discovery that NF−κB played a major role in protecting cells from apoptosis.

3.3 The IκB Family of Inhibitory Proteins

In most cell types, NF−κB is sequestered in the cytoplasm by a family of inhibitory proteins called IκB. Several members (Figure 6) have been identified including IκBα,

Figure 6. The NF-κB and IκB families of proteins. NF-κB transcription factors share a stretch of approximately 300 amino acids called the Rel homology domain which mediates DNA binding, dimerization and interaction with IκB members. The NF-κB family is comprised of five members; RelA, c-Rel and RelB which contain transactivation domains and p50 and p52 which are thought to be synthesized as precursor p105 and p100 respectively. The IκB family of proteins contains 5 members IκBα, IκBβ, IκBε, IκBγ and Bcl-3. p105 and p100 also function to inhibit NF-κB·DNA binding.

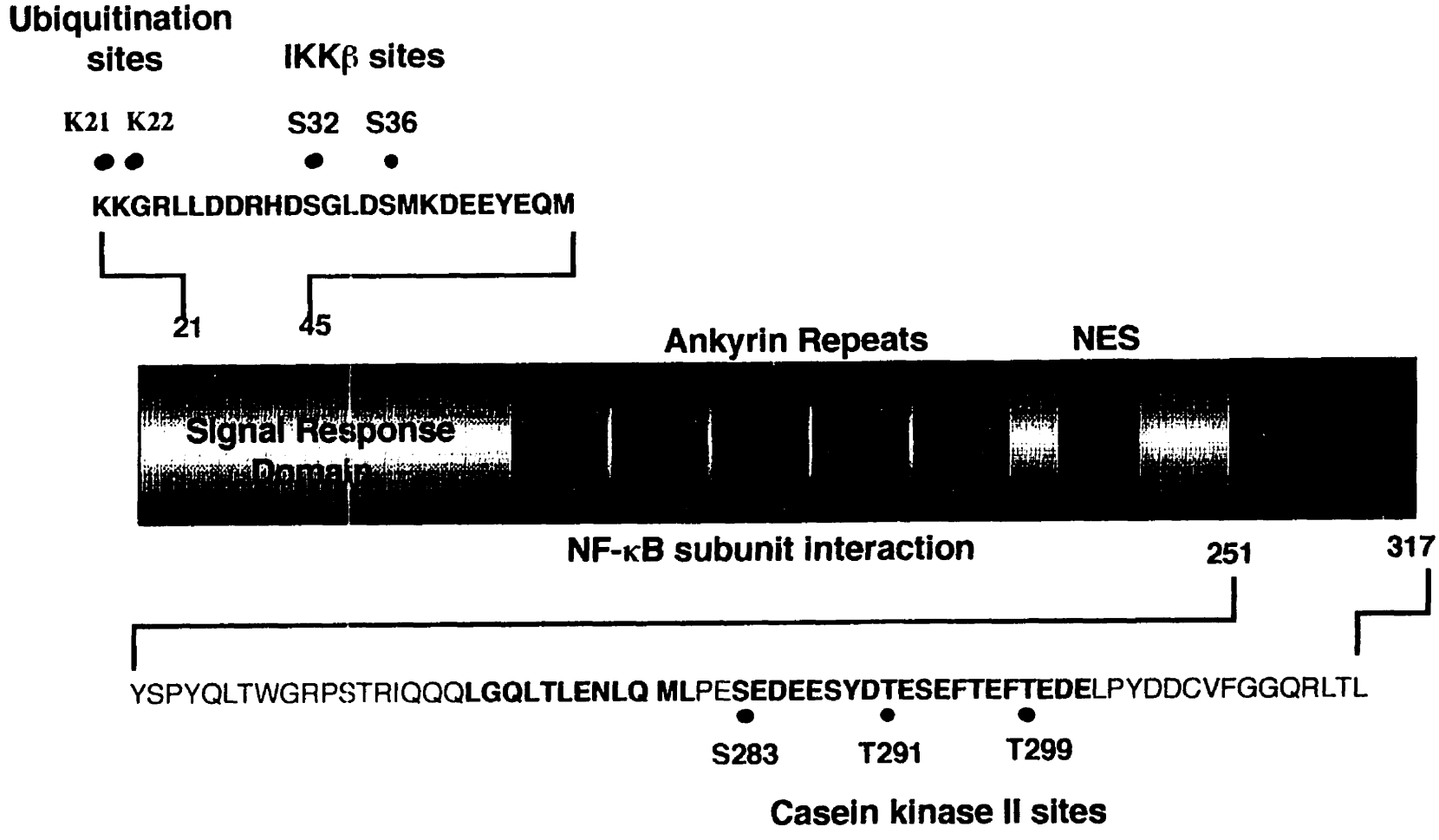

DNA BINDING
NON-DNA BINDING
INHIBITORS
Rel Homology
Transactivation
PEST
p65
IκBα
c-Rel
P
S/P
Bcl-3
Ankyrin Repeats
Proteolytic Cleavage
p105
Alternative splicing
p50
IκBγ
Proteolytic Cleavage
p100
p52
IκBβ
RelB
IκBε

IκBβ, IκBε, IκBγ and the proto-oncogene Bcl-3 (reviewed in 36,46). The precursor proteins p105 and p100 can also serve as functional IκB proteins, releasing NF–κB upon proteosome mediated proteolytic processing (327). Structurally, all IκBs contain six or seven repeats of a 33 amino acid domain termed the ankyrin motif (reviewed in 36,46) which is required for NF–κB interaction. IκB inhibits NF-κB transactivation by binding NF–κB and masking its NLS, preventing it from entering the nucleus.

3.3.1 *IκBα*

IκBα was the first identified IκB protein (28) and is the best characterized. IκBα contains an N-terminal signal response region, involved in inducer mediated degradation, six ankyrin repeats important for interaction with NF–κB family members, and a C-terminal PEST domain, which regulates its basal turnover (Figure 7) (39,264,392). Activating signals such as TNFα, lead to the activation of the recently identified I kappa B kinase (IKK) (73,120,293,380,462,484), which phosphorylates IκBα on serines residues 32 and 36, and targets the protein for ubiquitination (35,72,256,385,431,432). Ubiquitination is ATP dependent and involves the covalent attachment of multiple ubiquitin molecules to IκBα lysine residues 21 and 22. The 26S proteosome complex, which consists of a 20S multicatalytic protease complex and several regulatory proteins, recognizes and degrades the ubiquitinated protein, releasing NF–κB to translocate to the nucleus and activate regulated genes, including the *ikba* gene (MAD-3). Since IκBα is an NF–κB regulated gene, it is quickly resynthesized, forming an autoregulatory loop which functions to limit NF–κB activation. Mutation of serine residues 32 and 36 transforms IκBα into a transdominant repressor (44,239), able to almost completely block NF–κB·DNA binding by T cell activation signals such as PMA, TNF, LPS, HTLV-1 Tax and okadaic acid (36). Likewise, mutation of lysine residues 21 and 22 also generates a signal non-responsive form of IκBα (385), underscoring the importance of site specific phosphorylation and ubiquitination for IκBα degradation and NF–κB release.

Figure 7. Functional domains of IκBα. IκBα can be divided into three domains, the N-terminal signal response domain, the central ankyrin repeats and the C-terminal PEST domain. Phosphorylation of Ser32 and Ser36 marks the protein for ubiquitination on Lys 21 and Lys 22 and subsequent proteosome mediated degradation. The central ankyrin repeats mediate the interaction with, and sequestration of, NF-κB family members. A nuclear export sequence (NES) is found between the ankyrin repeats and the PEST domain and permits nuclear IκBα to remove DNA bound NF-κB and translocate it to the cytoplasm. The PEST domain, rich in proline, glutamic acid, serine and threonine residues, contains several casein kinase II sites which are important in regulating constitutive IκBα protein turnover.


Ubiquitination sites
IKKβ sites
K21 K22
S32 S36
KKGRLLDDRHDSGLDSMKDEEYEQM
21
45
Ankyrin Repeats
NES
Signal Response Domain
NF-κB subunit interaction
251
317
YSPYQLTWGRPSTRIQQQLGQLTLENLQ MLPESEDEESYDTESEFTEFTEDELPYDDCVFGGQRLTL
S283
T291
T299
Casein kinase II sites

Basal turnover of IκBα is regulated by the C-terminal PEST domain (45,264,392). Several Casein kinase II (CKII) phosphorylation sites exist in the PEST domain of IκBα and phosphorylation of these sites have been implicated in constitutive protein turnover (264,458). Several reports have suggested that C-terminal phosphorylation by CKII is also necessary for efficient inducer mediated degradation (45,412) although contrary findings have been reported (18,438).

3.3.2 *IκBβ*

IκBβ is similar to IκBα in function and structure (recently compared in 433). IκBβ contains an N-terminal signal response domain, six ankyrin repeats and an acidic PEST domain (182,427). IκBβ is inducibly phosphorylated by IKK on serine residues 19 and 23, and ubiquitinated and degraded by the multimeric proteosome complex (458). IκBβ differs from IκBα in that it requires basal phosphorylation in order to interact with NF–κB (85,288) and its kinetics of resynthesis after stimulus induced degradation are slower (427). IκBβ requires its C-terminal PEST domain for efficient proteolysis (179,449) and C-terminal CKII phosphorylation (85) to inhibit NF–κB binding.

Unlike IκBα, IκBβ is not an NF–κB regulated protein and is not rapidly resynthesized after inducer mediated degradation (427). Instead, IκBβ has been implicated in maintaining the persistent NF–κB activation caused by some inducers. This type of activation has been associated with the additional release of NF–κB from IκBβ complexes, its resynthesis as a hypophosphorylated form sustaining NF–κB activation (342,415). This theory is supported by transgene experiments where expression of constitutively active IκBβ (IκBβ that resists signal induced degradation) abolishes persistent NF–κB activation (23). Newly synthesized IκBβ reportedly binds NF–κB without masking its nuclear localization signal (85). NF–κB bound by hypophosphorylated IκBβ can then enter the nucleus and potentially bind DNA, protected from IκBα mediated dissociation.

Recently, two isoforms of IκBβ that differ in the C-terminal PEST domain as a consequence of alternative splicing, have been identified in human cells (191). The larger protein, approximately 43-kDa, is homologous to the mouse IκBβ while the 41-kDa protein is unique to human cells. The 43-kDa protein degrades upon stimulation and enters the nucleus when hypophosphorylated, while the 41-kDa protein is resistant to degradation by several inducers and is found only in the cytoplasm (191).

3.3.3 *IκBε*

IκBε is the newest IκB molecule to be cloned (reviewed in 431). Human IκBε is a 45-kDa protein, contains conserved serine phosphorylation sites but lacks a PEST domain. It associates preferentially with RelA and c-Rel containing dimers and is thought to regulate a late transient activation of genes. Mouse IκBε has also been cloned and found to function primarly in the cytoplasmic sequestration of RelA homodimers (397b).

3.4 Mechanism of NF–κB Activation

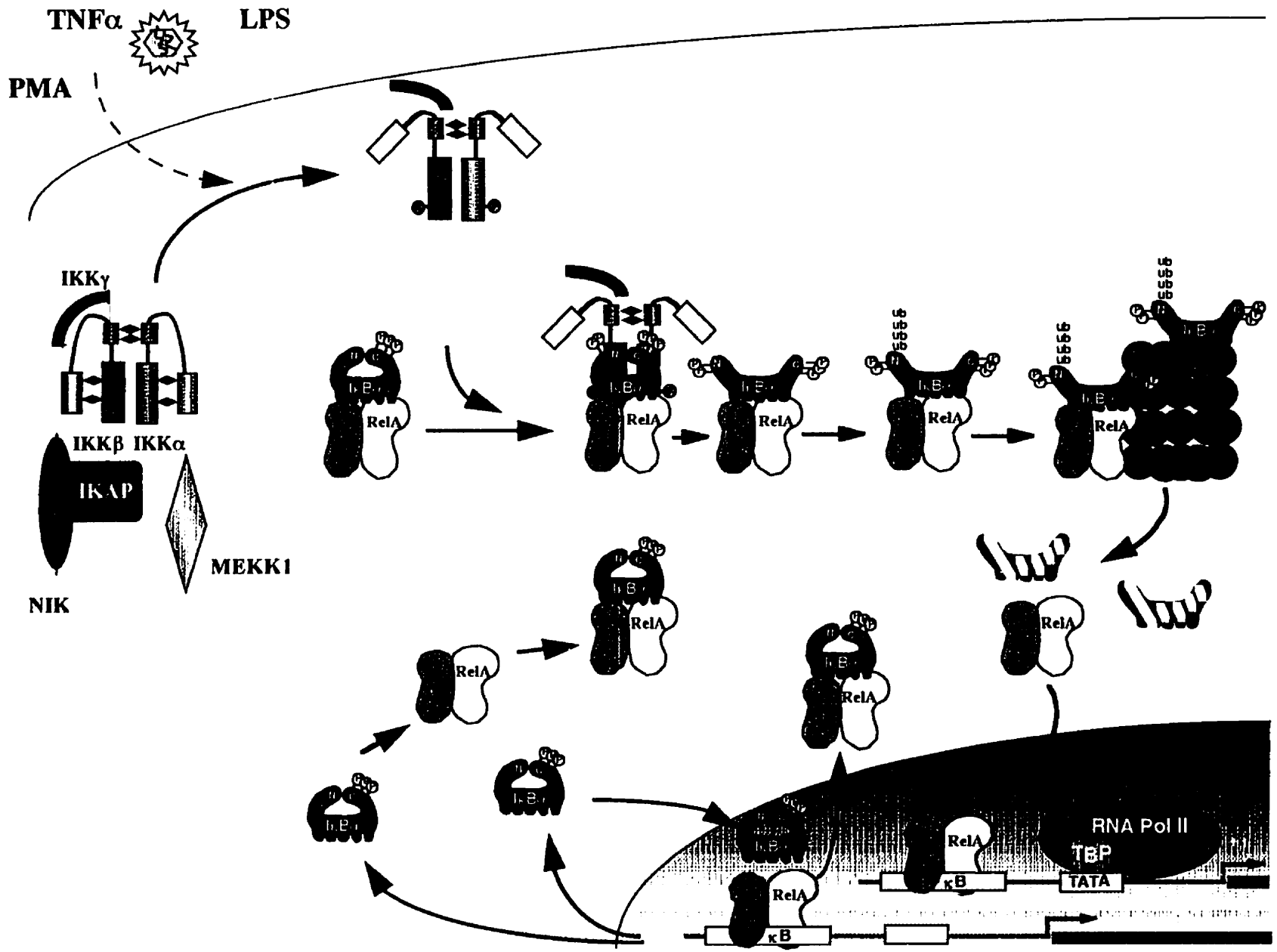
NF–κB is activated by many diverse stimulants ranging from bacterial and viral pathogens to a variety of cell damaging agents. Table 2 lists some of the many inducers of NF–κB activation. Common steps in these pathways are suggested by the broad inhibition of NF–κB achieved by certain inhibitors. Antioxidants, alkylating agents, and proteosome inhibitors prevent IκB degradation by a variety of inducers. Conversely, phosphatase inhibitors can lead to NF–κB activation in several cell types (291,292,398,411). Recently, many of the upstream players regulating NF–κB activation have been identified. Figure 8 illustrates the general mechanism of NF–κB activation.

Table 2. Agents and inducers of NF-κB·DNA binding activity.

Class	Inducing Agent
Bacterial Products	Lipopolysaccharide
	Exotin B
	Toxic shock syndrome toxin 1
	Muramyl peptides
Viruses	Human immunodeficiency virus type1 (HIV1)
	Human T-cell leukemia virus type 1(HTLV-1)
	Hepatitis B virus (HBV)
	Herpes simplex virus type 1 (HSV-1)
	Human herpes virus 6
	Newcastle disease virus
	Sendai virus
	Epstein-Barr virus (EBV)
	Adenovirus
Viral Products	Double stranded RNA
	Tax (HTLV-1)
	HBx (HBV)
	Epstein-Barr nuclear antigen2 (EBNA-2, EBV)
	Latent membrane protein (LMP, EBV)
Inflammatory cytokines	Tumor necrosis factor α (TNFα)
	Lymphotoxin (TNFβ)
	Interleukin-1
	Interleukin-2
	Leukotriene B4
	Lymphocyte inhibitory factor
T cell mitogens	Antigen
	Lectins (PHA, ConA)
	Calcium ionophores
	Anti-CD3
	Anti-CD2
	Anti-CD28
B cell mitogen	Anti-surface IgM
Protein synthesis inhibitors	Cycloheximide
	Anisomycin
Physical stress	UV light
	γ radiation
Oxidative stress	Hydrogen peroxide
	Butyl peroxide
Drugs	Okadaic acid
	Phorbol esters

Reproduced from reference (30).

Figure 8. The biochemistry of NF-κB activation. NF-κB is sequestered in the cytoplasm by inhibitory IκB proteins. Stimulation by a diverse collection of agents and pathogens including TNFα, PMA, LPS and viruses lead to the activation of signaling cascades that are believed to culminate with activation of the IKK complex. The IKK complex is composed of IKKβ, IKKα, IKKγ, IKAP and possibly NIK or MEKK1. Activated IKK phosphorlyates IκBα, which marks it for ubiquitination and subsequent proteosome mediated degradation. NF-κB is released and translocates to the nucleus where it transactivates κB responsive genes. Target genes are selectively regulated by the distinct transcriptional activation potential of different subunit combinations. The IκBα gene is regulated by NF-κB leading to its resynthesis. *De novo* synthesized IκBα can associate with free NF-κB sequestering it in the cytoplasm and can enter the nucleus removing DNA bound NF-κB, thereby establishing an autoregulatory mechanism by which NF-κB activation is limited.


TNFα
LPS
PMA
IKKγ
IKKβ
IKKα
IKAP
NIK
MEKK1
IκBα
RelA
κB
TATA
TBP
RNA Pol II

3.4.1 *Pathways involving IKK*

Several NF–κB inducing agents are known to activate the recently identified IKK complex (73,120,293,380,462,484), which phosphorylates both IκBα and IκBβ (280,406) on conserved serine residues. Phosphorylation targets these proteins for ubiquitination which in turn marks them for proteosome mediated degradation (see Figure 8). NF–κB is released and subsequently translocates to the nucleus where it activates κB responsive genes. Several pathways are thought to converge at the level of IKK activation, implicating this complex as a critical regulator of NF–κB transcriptional regulation.

The multimeric IKK complex includes two subunits, IKKα and IKKβ, which are responsible for phosphorylating IκB molecules. Several other components of the IKK complex have been identified including the regulatory subunit NF–κB essential modulator (NEMO, also called IKKγ) (369,469) and a scaffolding protein IKK associating protein (IKAP) (91), which binds the IKK subunits and together with NF–κB inducing kinase (NIK), assembles them into an active kinase complex. The IKK complex is rapidly stimulated by TNF, IL-1 and PMA, although the mechanism of activation requires further elucidation.

Recently, NIK was found to activate IKKα directly (266), confirming earlier reports that NIK co-expression leads to IKKα phosphorylation (380). MEKK1 has also been found tightly associated in the IKK complex (293) and has been shown to stimulate IKK activity (253). NIK and MEKK1 are attractive candidates for regulating IKK since both IKKα and IKKβ contain a canonical MAPKK activation loop motif (SxxxS) (293); further studies are required to determine whether these kinases are essential upstream regulators.

3.4.2 *Other mechanisms of NF–κB activation*

p105 is phosphorylated by TPL-2 (49) and degraded by the proteosome in a ubiquitin

dependent fashion (96,327), releasing a p50 containing dimer of NF–κB. This pathway may be particularly important in HIV-infected monocytes which exhibit increased processing of p105 (287). HIV-1 protease directly cleaves p105 (361) and hence may contribute to NF–κB activation, creating an environment conducive to high levels of HIV-1 gene expression. A similar pathway likely exists for p100 but remains to be described.

Several other kinases have been implicated in NF–κB signal transduction pathways. The Ras/Raf pathway can target IκBα (56) and induce NF–κB mediated gene transcription (143). PKC has been implicated in taxol induced NF–κB (403) and PKCγ specifically, in PMA induced NF–κB activation (118,119). p90rsk activates NF–κB via IκBα phosphorylation (389) and the double stranded RNA protein kinase PKR plays a role in viral induced NF–κB activation (236,237). The relative contribution of these kinases is unknown and remains an area under active investigation.

Phosphatase inhibitors lead to NF–κB activation, suggesting that phosphatases are important *in vivo* regulators of the NF–κB pathway. They may regulate kinase pathways involved in NF–κB activation, or may directly dephosphorylate IκB or both. Although IκBα is not thought to be tyrosine phosphorylated (one report suggests that it may (203)), tyrosine phosphatase inhibitors can induce an HIV-1 LTR luciferase construct in an NF–κB dependent manner (38). In addition, the $Ca2^{+}$ phosphatase, calcineurin, seems to be important for activation of NF–κB in T cells (154). Inhibitors of PP1 and PP2 phosphatases, such as okadaic acid, potently activate NF–κB and studies have found a direct role for PP2 in the dephosphorylation of IκBα (302,411).

Reactive oxygen intermediates or (ROIs) are also important mediators of NF–κB activation. The evidence for this statement is threefold. Most importantly, antioxidants such as N-acetyl cysteine (NAC) or pyrrolininedithiocarbamate (PDTC) can block IκBα

degradation and NF–κB activation (254,489). Treatment of some cells with H_2O_2 can induce NF–κB and many of the known inducers of NF–κB generate ROIs. However, downstream effectors of ROIs have not been definitively identified.

3.4.3 *NF–κB subunit phosphorylation*

In addition to the phosphorylation of p105 and p100 which targets them for proteolytic processing, several NF–κB proteins are inducibly phosphorylated upon cell stimulation. RelA is phosphorylated upon TNFα induction (121) at serine residue 529 (445b). Phophorylation increases its transcriptional activity but does not affect DNA binding or nuclear translocation. Although other family members are known to be phosphoproteins, the effect of phosphorylation on other family members have not been studied.

3.5 TNFα Signaling Pathway

Tumor necrosis factor α (TNFα) is a multifunctional cytokine; it can induce cell proliferation or cell death. TNFα mediates its cellular functions through two distinct receptors, TNFR1 (also called p55 or p60) and TNFRII (p75 or p80), which are part of the TNF/nerve growth factor (NGF) receptor superfamily (reviewed in 33). Other family members include the lymphotoxin β (LTβ) receptor, Fas (CD95, Apo-1), CD40, CD30, CD27, p75 NGF receptor, OX-40 and death domain receptor 3 (DR3). These receptors share similar extracellular domains but their cytoplasmic domains are quite distinct. TNF/NGF family members do not exhibit any intrinsic enzymatic function and signaling therefore results entirely from the recruitment of adapter molecules. Several family members induce NF–κB in addition to activating other pathways.

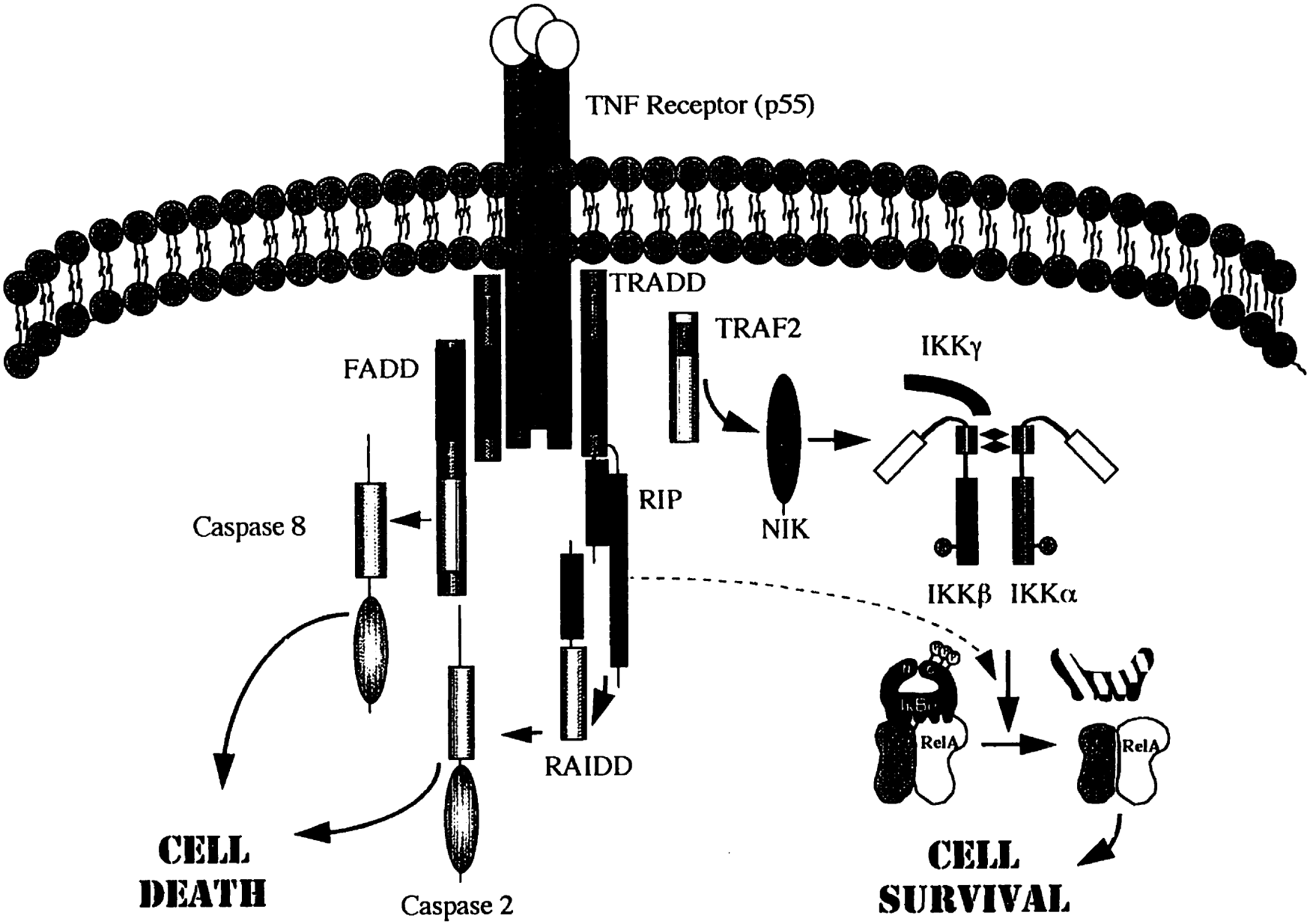
TNFα activates NF–κB (illustrated in Figure 9) within minutes of ligand binding and can induce apoptosis upon prolonged stimulation (reviewed in 107). TNFR1 mediates most

of the known TNFα cellular responses including NF–κB activation and apoptotic signaling. Ligand binding induces receptor oligomerization on the cell surface and is assumed to bring many of the molecules which associate with the cytoplasmic domain, into close proximity. The cytoplasmic tail of TNFR1 contains an 80 amino acid death domain motif which interacts with the TNF receptor associated death domain (TRADD) protein (198), a signaling molecule with a similar death domain motif. Overexpression of TRADD activates NF–κB and induces apoptosis, implicating it as an important mediator of TNFα signaling.

Fas associated death domain (FADD) protein interacts with the adaptor molecule TRADD as well as the Fas receptor (reviewed in 33). FADD associates with caspase 8 (FLICE, MACH) and signals apoptotic cell death when activated. Transdominant mutants of FADD prevent TNFα induced apoptosis but do not affect TNFα activation of NF–κB, suggesting that FADD plays a central role in cell death but is not involved in mediating TNFα induced NF–κB activation.

TRADD also interacts with the TNF receptor associated factor 2 (TRAF-2), a member of the TRAF family of proteins, via its N-terminus. TRAF-2 activates NF–κB (as do many of the other TRAF family members), but does not induce apoptosis, placing it downstream of TRADD in the TNFα signaling cascade. TRAF-2 interacts with NIK (402), which as previously mentioned, can activate IKK. TRAF-2 is however not the only mediator of TNFα induced NF–κB activation since TRAF-2 knock-out mice have functional TNFα induced NF–κB activation (476).

Figure 9. TNF receptor I signaling. Binding of TNFα induces trimerization of TNFRI inducing receptor activation. The TNFR activated death domain protein, TRADD, is recruited to the receptor and interacts with TNFRI via their shared death domain motifs. TRADD interacts with TNF receptor associated factor-2 (TRAF-2) protein and receptor interacting protein (RIP) kinase, and both these proteins activate NF-κB. TRAF-2 activates the NF-κB inducing kinase (NIK) which phosphorylates and activates the IKK complex. Activated IKK phosphorylates IκB proteins leading to the release of NF-κB proteins. These proteins translocate to the nucleus and activate genes important for cell survival. The mechanism of RIP induced NF-κB activation does not require its kinase function but remains unknown. RIP also interacts with the RIP-associated ICH1/CED3-homologous protein with death domain (RAIDD) which activates death signaling pathways via caspase 2. Death signaling is also activated by pathways initiated by the Fas activated death domain protein (FADD). FADD is recruited to the TNFRI upon receptor oligimerization and activates the associated FADD like ICE (FLICE) enzyme, now called caspase 8. Caspase 8 processes downstream caspases and leads to cell apoptosis. The fate of the cell is hence determined by the champion of conflicting pathways: anti-apoptotic NF-κB signaling versus pro-apoptotic caspase activation.


TNF Receptor (p55)
TRADD
TRAF2
IKKγ
FADD
RIP
NIK
Caspase 8
IKKβ
IKKα
RAIDD
RelA
RelA
CELL DEATH
Caspase 2
CELL SURVIVAL

A 74-kDa protein, harboring an N-terminal kinase domain and a C-terminal death domain, was also found to interact with TNFRI (reviewed in 107). Receptor interacting protein (RIP) was first identified by its interaction with Fas and by a weak interaction with TNFRI (407). This interaction was later shown to occur through TRADD. RIP has two binding sites for TRAF-2 and can associate with TRAF-1 and TRAF-3. Overexpression of RIP or RIP lacking its kinase domain, induces apoptosis and NF–κB activation (197). NF–κB activation requires only the intermediate region of RIP, but can be potentiated by the kinase and death domains.

RIP association with TNFR1 via TRADD is TNFα dependent (197). TRADD mutants which do not activate NF–κB or induce apoptosis, fail to recruit RIP to the TNFR. RIP may therefore play an important role in NF–κB activation. RIP likely serves additional functions: its kinase domain is dispensable for NF–κB activation and it binds Fas via FADD which does not usually activate NF–κB.

Association of the aforementioned proteins has only been seen in overexpression studies: no interaction of endogenous TNFRI with TRAF-2, TRADD or FADD has been demonstrated to date. The recruitment process of TNFR1 interacting proteins has eluded discovery. Other kinases such as the serine/threonine kinase TNFR associating kinase, p60TRAK (108), which directly binds the TNFR1 tail, may be involved in this process.

3.6 Virus Induced NF–κB Activation

NF–κB is also activated by viral infection. This activation may serve two functions: NF–κB activation mediates the immune response to the invading pathogen and in several instances promotes viral replication. Many viral products activate NF–κB, including the HTLV-1 Tax protein (20,216,240), the HIV-1 Tat protein (384) and the Epstein Bar

virus (EBV) latent membrane protein-1 (LMP-1) (134,188). Activation in these cases is self-serving, viral replication is enhanced by NF-κB activation.

Tax induces NF−κB by stimulating MEKK-1 which activates the IKK complex (340,478). Tax may also directly interact with DNA bound NF−κB to stimulate transcription (417). Other studies have found that Tax binds IκBα, promoting IκBα degradation via the proteosome (339). NF−κB activation may promote HTLV-1 pathogenesis via the upregulation of several cellular factors such as IL-2, IL-2Rα and IL-6 (305) which induce HTLV-1 replication.

Activation of NF−κB by Tat is less clear. Recombinant Tat protein can increase LTR CAT expression and this phenomenon is dependent on NF−κB (44). IκBα is degraded and agents that block IκBα prevent Tat activation of NF−κB. Tat activation of TNFα production is also mediated by NF−κB (455) and hence Tat may activate a regulatory loop where NF−κB activation is perpetuated. Tat exerts a variety of effects on cell growth and it is postulated that several of the cytokine like effects of this viral protein are mediated through NF−κB (12).

NF−κB is more broadly activated by viral infection and its activation is necessary for the activation of immune response genes. NF−κB is necessary for the activation of IFN-β gene expression (192), a critical mediator of the IFN anti-viral response. The positive regulator domain II (PRDII) element of the IFN-β gene binds NF−κB in association with the high mobility group protein HMG I(Y) (425). Mutation of either the NF−κB site or the HMG site blocks IFN-β activation. It has been proposed that formation of a higher order complex containing NF−κB, HMG, IRF-1 and b-Zip proteins (ATF-2 and c-Jun) is required for IFN-β gene expression (252). NF−κB also activates several adhesion molecules, the peptide transporter TAP1 gene and the proteasome subunit LMP2, the

MHC class II invariant chain gene, the inducible nitric oxide synthase gene (iNOS), as well as numerous cytokines and growth factors, such as TNFα, IL-2, IL-6, and G-CSF, all of which are important in immune responses (reviewed in 30).

The general mechanism of viral induced NF–κB activation is not clear. HIV viral fusion activates NF–κB and this process requires CD4 (58); other viruses may activate similar membrane proximal signaling cascades which activate NF–κB. NF–κB is also activated by synthetic dsRNA suggesting a common mechanism may be employed by dsRNA viruses (318). Cells expressing antisense PKR and PKR knock out cells are dysfunctional for dsRNA induced NF–κB, implicating PKR as mediator of dsRNA induced NF–κB activation (237,474).

3.6.1 *Role of PKR*

PKR activation of NF–κB may directly target IκBα since *in vitro*, PKR can phosphorylate IκBα (236). The *in vivo* ability of PKR to phosphorylate IκBα has not been shown and it is possible that PKR exerts its effect upstream, inducing a kinase cascade that leads to NF–κB activation.

The interferon inducible, dsRNA activated protein kinase PKR, plays a variety of roles in the regulation of translation, transcription and signal transduction pathways (reviewed in 88,351). PKR mediates some of the antiviral activities of the IFNs including inhibition of translation. PKR inhibits protein synthesis by phosphorylating the eukaryotic initiation factor eIF2α, PKR's best known and first identified substrate. Transcriptional control by PKR is mediated in part by NF–κB; this is suggested by co-transfection studies in which wild type PKR expression enhances expression of genes containing NF–κB response elements (295). More recently, PKR has been implicated in dsRNA and TNFα signaling of apoptosis, supporting earlier studies finding PKR to be a tumor suppressor (232).

PKR has a conserved kinase domain in its C-terminus and two dsRNA binding modules in its N-terminus (reviewed in 351). DsRNA activation induces PKR autophosphorylation or more likely transphosphorylation in newly associated PKR dimers. Phosphorylation occurs at multiple sites and is obligatory for its ability to phosphorylate other substrates (366). Once activated, the kinase activity of PKR for other substrates is independent of dsRNA and thought to be inhibited only by dephosphorylation of sites critical for PKR activity.

The type of dsRNAs that activate PKR requires further investigation, although several candidates, including the HIV-1 TAR element, have been identified (375). Although some groups have disputed TAR's ability to activate PKR, experiments employing synthetic RNAs have provided evidence that TAR at low concentrations, is a *bona fide* activator of PKR (285). Interestingly, Tat can also interact with PKR and is phosphorylated by PKR (57,290). Tat can inhibit PKR's ability to phosphorylate eIF2α implying that HIV-1 may be able to overcome the block to protein translation mediated by PKR (57).

Several other viruses have developed strategies to prevent PKR mediated inhibition of translation, arguing a role for PKR in general host defense mechanisms (reviewed in 88). Adenovirus small VAI RNA and vaccinia virus K3L protein both inhibit PKR activation. Cellular inhibitors of PKR include p58 and the TAR binding protein. Although the function of the latter is unknown, it is believed that p58 may be important for regulating PKR function induced by cellular stresses such as calcium influx and heat shock. These viral inhibitors may also affect PKR mediated regulation of cell growth and apoptosis.

4.0 IMMUNOLOGICAL EFFECTS OF HIV-1 INFECTION

HIV-1 infection leads to profound immune deficiency and eventual destruction of the immune system causing death. A long clinical latency period misleads us to believe that the immune system has gained control over the persistent infection caused by HIV-1 but the onset of opportunistic infections reminds us that the host is fighting a losing battle. A central theme in HIV immunopathogenesis is the ability of the virus to undermine the pathways designed to protect the host against invading pathogens. HIV invades lymph nodes, establishing itself as a permanent resident. The function of the lymph node is to filter antigen from the peripheral blood and trap it in the follicular dendritic cell (FDC) network, allowing germinal center B cells and $CD4^+$ T cells in the paracortical areas to undergo antigenic stimulation, initiating a protective immune response. HIV-1 is similarly trapped by the FDC network and initially, the functionally intact lymph node facilitates antigen presentation and generates an anti-HIV response (330). This response requires the continual recruitment and subsequent activation of peripheral blood lymphocytes. Trapped HIV-1 infects susceptible target cells, thus permitting the ongoing infection of newly recruited cells (421). In addition, HIV has an intrinsic ability to mutate rapidly, which allows the virus to evade both humoral and cell mediated immune responses and escape containment.

4.1 HIV-1 and T cells

The fundamental abnormality in HIV-infected individuals is a progressive decrease in $CD4^+$ T lymphocytes (245). It is believed that 2×10^9 $CD4^+$ T cells are destroyed each day in late stages of disease (195,447). $CD4^+$ T cells are critical mediators of immunological responses against a variety of pathogens as well as malignant tumors. They interact with B cells, natural killer cells, cytotoxic T cells and monocyte/

macrophages primarily through the release of soluble factors and, to a lesser extent, cell to cell contact. $CD4^+$ T cells affect the growth and differentiation of lymphoid cells and hematopoietic cells as well as the function of non-lymphoid cells. Hence disturbances in $CD4^+$ T cell function, quantitative or qualitative, can have profound effects on the functioning of the immune system (135).

The drastic decline in circulating $CD4^+$ T cells induced by HIV-1 during primary infection, may result from direct killing of infected and non-infected cells, the redistribution of circulating $CD4^+$ T cells, or both (429). Since 98% of the total lymphoid pool resides in lymphoid organs compared to only 2% in the periphery, it is possible that decline in circulating $CD4^+$ T cells is the result of a redistribution of these cells from the peripheral blood compartment to the lymphoid tissue (274). $CD4^+$ T cells may be recruited to lymph nodes to participate in an immune response against HIV, becoming infected and destroyed in the process. This could explain why even though $CD4^+$ T cell numbers rebound after the primary infection, their numbers are generally lower than pre-infection levels.

During the asymptomatic stage, $CD4^+$ T cells slowly decline and functional defects in immune responses are observed. Although relatively few peripheral blood cells are productively infected, recent studies indicate that high levels of virus production and T cell death persist in the lymph nodes of asymptomatic individuals. $CD4^+$ memory T cells are particularly affected. Total numbers may also decrease due to impairments in T cell regeneration. Defects in hematopoietic regeneration have been reported in HIV(+) individuals, manifesting as thrombocytopenia, granulocytopenia, anemia, lymphopenia and pancytopenia (95). Although some of these conditions may be the result of opportunistic diseases, many abnormalities appear to be HIV related.

Bone marrow precursor cells represent a major source of progenitors for $CD4^+$ T cells and lack of replenishment may in part be due to HIV-1 infection of bone marrow progenitor cells. Progenitor $CD34^+$ bone marrow cells can indeed be infected *in vitro* (148,229) although *in vivo* results have been inconsistent (147,301,408). The impairment of $CD4^+$ T cell regeneration in HIV(+) individuals, may also result from infection of precursor cells in the thymus. Immature $CD4^+/CD8^+$ thymic lymphocytes, human fetal thymocytes, mature thymocytes, as well as immature $CD3^-CD4^-CD8^-$ intrathymic T cell precursors can all be infected with HIV-1 *in vitro*. Experiments conducted in a severe combined immunodeficiency (SCID) mouse model of human fetal thymic transplantation, support this theory. In these studies, HIV-1 could infect multiple subsets of T cells as well as thymic epithelial cells, strongly suggesting that this phenomenon occurs in HIV-infected individuals.

Responses to a range of different antigens (244), T cell colony formation, expression of IL-2 receptors, and IL-2 production are defective in $CD4^+$ T cells from HIV-1(+) individuals. Although preferential infection of $CD4^+$ memory T cells (387) is the likely cause of the deficient antibody response, mechanisms explaining other defects have yet to be clearly identified. Importantly, T cell dysfunction also occurs in non-infected cells. Incubation of T cells *in vitro* with non-infectious virus, inactivated virus or viral proteins gp120 and Tat, suppresses T cell responses to mitogens and antigens (387,450). This could explain the general immunosuppression seen in HIV-infected individuals even though only a fraction of T cells are productively infected.

Although $CD4^+$ T cell responses are compromised, infected individuals mount a strong $CD8^+$ CTL response to HIV (289). CTL responses peak as viremia begins to fall and this precedes the detection of any neutralizing antibody (233), suggesting that CTLs are important in containing HIV infection. Supporting this role are the observations that

CTLs can inhibit HIV replication in cell lines *in vitro*, both by direct killing and the release of chemokines and other cytokines (472). The strong CTL response likely exerts a selective pressure on the virus causing it to mutate to evade CTL mediated death.

In advanced disease where $CD4^+$ T cells fall below 200 cells/ml, infected individuals are at great risk of developing a number of opportunistic infections and malignancies (138). Examination of lymph nodes by electron microscopy reveals involution of germinal centers and degeneration of the FDC network (92). The FDC have diminished ability to trap virus and immune responses to HIV deteriorates. As a result, HIV virus in peripheral blood increases. The lymph node virtually disintegrates, debilitating immune responses and hence permitting opportunistic infections to take hold. This results in the stimulation of cytokine production, which activates HIV replication, further compromising the immune system. The cytokines also activate $CD4^+$ T cells, allowing the remaining uninfected $CD4^+$ population to become permissive for HIV-1 infection.

The course and pace of HIV disease progression is variable among individuals. Several virologic and immunologic mechanisms influence the clinical progress of HIV induced disease (171). HIV rapidly establishes a pool of latently infected cells which are long lived (estimated half life of 3-6 months), even after suppression by HAART (highly active antiretroviral therapy), posing a serious problem for long term disease control. The ability of HIV to mutate rapidly, allows it to escape immune control and the trapping of infectious virus in the FDC network of lymphoid tissue permits a continuous reservoir of virus for the *de novo* infection of cells that enter or reside in the lymphoid tissue (130,329,330). HIV infection further induces several immunologic changes that enhance its replicative potential. The deletion of HIV specific $CD4^+$ clones and HIV specific $CD8^+$ CTL permits HIV to escape immune control. In addition, HIV specific activated CTLs are more frequently found in the periphery than in lymph nodes (171); this could

explain ineffective elimination of HIV virus from lymph nodes and allow virus to spread in the early stages of infection.

4.2 HIV-1 and Monocytes/Macrophages

Cells of the monocyte lineage are $CD4^+$, express HIV chemokine coreceptors, and are hence permissive to HIV infection. Although their relevance in HIV-1 infection has been disputed (331), numerous observations suggest that they are indeed important mediators of HIV-1 pathogenesis. The observations that virus isolates during initial infection are overridingly macrophage tropic and individuals homozygous for a CCR5 deletion mutant that precludes receptor surface expression, exhibit near complete protection from HIV-1 infection, support the contention that monocytic cells are likely mediators of early HIV-1 infection (190). These cells are long-lived and capable of producing high titers of virus (336) particularly when stimulated.

Monocytes and macrophages are present in the genital tract (89,234) and are thus directly exposed to, and likely infected by, HIV-1 during sexual transmission (341). Recent studies have shown that HIV-infected primary monocytes can travel between epithelial cells, secreting virus which subsequently infects the $CD4^-$ epithelial cells (341). In addition, mouse macrophages stained with supravital dyes and inoculated intra-vaginally, could rapidly penetrate vaginal epithelium, traveling through the underlying connective tissue and into lymph nodes (341). Monocytic cells may therefore serve to spread the initial inoculum of virus by infecting epithelial cells and/or by trafficking virus to lymph nodes where a ready supply of permissive cells are located.

Dendritic cells, which can be generated *in vitro* from blood monocytes in the presence of GM-CSF and IL-4 (336), are also likely one of the first cell types infected by HIV-1.

Dendritic cells exhibit reduced antigen presenting abilities upon exposure to HIV-1 and eventually become targets for anti-HIV CTL (231). Loss of dendritic cell function and cell numbers upon exposure to HIV-1 may result in lower levels of antigen dependent T cell stimulation and contribute to T cell depletion in HIV-1 infection.

Cells of the monocyte/macrophage lineage have also been implicated in HIV neurological dysfunction. Macrophages and microglia (the resident macrophages of the brain), are the major types of HIV infected cells in the brain and likely the main cause of AIDS dementia complex (ADC). SCID mice, inoculated with purified virus infected human monocytes, provide evidence that macrophages secrete neurotoxins which cause neuronal injury and death (338). Soluble factors secreted by macrophages also induce astrocyte cell death (319). Although not confirmed, the production of quinolonic acid, platelet activating factor, arachadonic acid and cytokines such as TNFα by macrophages and microglia may be involved in mediating neuronal and astrocytic cell destruction (103).

Macrophages from HIV-infected individuals are generally impaired and cannot effectively protect the host from infection by intracellular parasites (345). This likely involves depression of monocytic cell function by HIV-1 in both infected and uninfected cells. The gp120 protein inhibits IFNα production, providing one explanation for the diminished macrophage antiviral activity seen in HIV-infected individuals (345). Free gp120 protein is readily found and the decreased IFN secretion precludes the establishment of an anti-viral state in uninfected cells, suggesting that macrophages need not be HIV-infected to be functionally compromised. This inability to effectively clear infections may be particularly relevant in HIV-1 pathogenesis.

Although their antiviral properties may be affected, HIV-infected macrophages likely account for the increased level of HIV-1 viremia seen in late stage infections where they

account for a greater proportion of infected cells due to declining $CD4^+$ T cell numbers. They have been identified as the major producer of HIV-1 in lymph nodes of individuals co-infected with opportunistic pathogens (324). The burst of HIV-1 replication is independent of actual macrophage co-infection; both *Mycobacterium kansasii* which infect macrophages and *Pneumocystis carinii* which do not infect macrophages, resulted in greatly elevated levels of HIV-1 production. In contrast, in lymph nodes that were not co-infected with an opportunistic pathogen, few HIV-infected cells of the monocyte lineage were present. Therefore, opportunistic pathogen co-infection can recruit HIV-infected and infectable macrophages to lymph nodes, stimulating viral expression and promoting viral spread. These observations suggest that a large reservoir of HIV-infected macrophages exist in the host that can, under the appropriate conditions, become a major source of virus.

4.3 Modulation of HIV-1 by Host Factors

Host factors play a major role in affecting the course of HIV disease (136). Although HIV induces AIDS in the majority of individuals infected, some persons develop a non-progressive HIV disease (long term non-progressors) (255,261,390). Certain HLA class 1 haplotypes are found more often in long term non-progressors than in the general population (218), suggesting that immune control of HIV infection is possible in individuals who can mount a more effective cell mediated response due to their HLA genetic background. In addition, immune responses to HIV infection can affect disease progression (328). Individuals who mount a cytotoxic $CD8^+$ T cell response that is restricted to a single clonal expansion, usually progress very rapidly to AIDS, while those experiencing a CTL multi-clonal expansion generally exhibit a slower progression (328).

HIV replicates more quickly in activated cells (61,482), and events that lead to cellular

activation of the immune system exacerbate viral load. Exogenous stimuli, such as opportunistic infections (87,167) substantially increases viral levels which return to baseline following resolution of the infection (167). HIV infection of immune system cells, is itself a potent and persistent cause of immune activation. Several endogenous cytokines released by activated cells have a profound effect on HIV disease progression and their expression is enhanced in HIV infected people (348). Numerous cytokines induce HIV expression including IL-1β, IL-2, IL-3, IL-6, IL-12, TNFα and TNFβ and colony stimulating factors M-CSF and GM-CSF (137). IFNα and IFNβ suppress HIV replication whereas TGF-β, IL-4, IL-10, IL-13 and IFNγ either induce or suppress HIV expression, depending on the cell system (348). Studies in peripheral blood mononuclear cells (PBMC) and lymph node mononuclear cells indicate that HIV expression is intimately related to cytokine expression. Inhibition of TNFα, IL-1β or IFNγ signaling by receptor antagonists or antibody neutralization, can drastically and sometimes completely suppress viral replication (227,228).

The mechanism of cytokine stimulation of HIV replication is best understood for TNFα, the most potent of the HIV inducing cytokines (348). TNFα activates the cellular transcription factor NF−κB, which in addition to inducing HIV LTR mediated transcription (125,349), also induces expression of numerous other genes which promote HIV expression (50).

Inhibition of HIV infection is mediated by several known soluble factors. Levy initially reported the suppressive effects of soluble factors released by $CD8^+$ T cells. Searching for the identity of these factors lead researchers to the β-chemokines RANTES, MIP-1α and MIP-1β (90), ligands of the M-tropic coreceptor CCR5. The β-chemokines effectively inhibited HIV infection of T lymphocytes of M-tropic strains but could not account for all the suppressive effects of $CD8^+$ T cell supernatants (124,307). Infection by T-tropic

strains of HIV could successfully be blocked by stromal derived factor (SDF-1), ligand of the CXCR4 coreceptor (53,321), implying that individuals with high levels of these chemoattractants might be partially protected from progression of HIV disease.

Chemokine ligands are proinflammatory proteins that induce leukocyte migration and activation (31), and can compete with HIV-1 for receptor usage. Several ligands are able to bind CCR5; these are the CC or β chemokines RANTES, MIP-1α and MIP-1β mentioned above. These chemokines exert suppressive effects upon HIV-1 replication of M tropic viruses, but can also enhance HIV replication of both X4 and R5 viruses at high concentrations. CXCR4 has only one known ligand, SDF-1 (460), which can accordingly inhibit HIV-1 infection of T-tropic strains. A polymorphism that leads to increased SDF-1, delayed the onset of AIDS and greatly increased the time to death (15), underscoring the role played by CXCR4 tropic viruses particularly in late stage disease. It appears that chemokine receptor G protein signaling is not required for viral entry although binding of envelope glycoprotein induces activation of the heterotrimeric G proteins (110,451). These signals may be important for post-entry events and retroviral replication.

Individuals carrying genetic variants of the HIV coreceptors CCR5 (111,202,297) and CCR2 (362,401) genes, also have a slower progression of HIV disease. A 32 base pair deletion in the CCR5 results in a truncated protein that is not expressed on the surface. Dramatically, individuals carrying homozygous mutations in this gene are almost completely resistant to HIV infection (111), highlighting the role played by M-tropic strains of HIV in establishing a productive infection.

4.4 Pathogenicity of HIV-1: Potential Mechanisms

4.4.1 *Direct cytopathic effects*

Virus replication continues throughout the course of HIV disease indicating that direct cytopathic effects of viral replication may contribute to the progressive loss of $CD4^+$ cells and the accompanying immune destruction. *In vitro* experiments have shown that HIV-1 infection of $CD4^+$ cells leads to extensive cell death. Several mechanisms for direct killing can be postulated. The massive budding of HIV-1 progeny virion may lead to loss of cell integrity and consequent cell death. Accumulation of integrated viral DNA has also been suggested to lead to cell death (332,368).

Interaction of HIV-1 envelope glycoprotein with the CD4 molecule present on the surface of other $CD4^+$ cells can lead to cell fusion and the formation of mutinucleated cells or syncitia. A few infected cells could therefore lead to the destruction of numerous uninfected $CD4^+$ cells (65,332). This may be particularly important in late stage disease when the *in vitro* phenotype of virus isolates changes from NSI (non-syncitia inducing) to SI (syncitia inducing) (423). Downregulation of the CD4 antigen by viral products gp160 (97,98), Nef (160,161,298,399) and Vpu (459) may also disrupt $CD4^+$ T helper (Th) cell function (98).

4.4.2 *Indirect virus mediated suppression of immune cell function*

Immune cell dysfunction may arise by soluble factors such as cytokines that affect the ability of uninfected cells to mount an immune response. CD4 binding of free gp120 and/or gp120 immune complexes has been posited to induce T cell anergy and depression of monocytic cell function (345), preventing functional immune responses (332). The initial inhibition of IFN production experienced during the asymptomatic stage may preclude containment of the virus. This would diminish immune clearance of HIV-1 and

HIV-infected cells, allowing the virus to disseminate around the body.

4.4.3 *Apoptosis*

Apoptosis has been suggested as both a direct and indirect method adopted by HIV-1. Even very early in infection, HIV-infected PBMC undergo *ex vivo* apoptosis more readily than uninfected cells (170). The importance of HIV-1 induced apoptosis has been contested on the grounds that fewer cells are detectably infected and consequently dying by apoptosis, than the numbers required to account for the T cell loss seen in HIV-1 infection. However, new methodologies have allowed the direct comparison of HIV-1 infection and induction of apoptosis. In the periphery, apoptosis can be seen in many, but not all, T cells which are HIV-infected. Apoptosis is at the same time occurring in many cells which are not positive for HIV-1 infection, arguing that direct and indirect induction of apoptosis may be an important problem in HIV-1 infection.

5.0 APOPTOSIS AND VIRAL INFECTION

Many viruses induce apoptosis in susceptible host cells and HIV-1 is no exception. Apoptosis may result from direct viral induction or as a host mediated defense mechanism. Viruses generally induce apoptosis of infected cells to propagate viral spread; CTLs induce apoptosis in infected cells to eliminate viral spread. Apoptosis can also occur in non-infected cells as an indirect consequence; secreted viral proteins may induce apoptosis or perturb normal cell signaling pathways, activating cell death.

The fact that numerous viruses have developed strategies to counter host induced apoptosis emphasizes the importance of this mechanism in immune anti-viral responses. The cowpox virally encoded CrmA protein is a potent inactivator of caspases (354); a number of viruses encode proteins that resemble Bcl-2, others encode proteins that

inactivate p53 (reviewed 440). Baculoviruses have been found to encode two new classes of apoptotic inhibitors p35 and the inhibitors of apoptosis proteins (IAP). Mammalian homologs of IAP have recently been identified and shown to protect cells from various inducers of apoptosis including TNFα (86,445).

CTLs can recognize and induce apoptosis of virally infected cells (283) by several distinct pathways (reviewed in 156). CTLs express FasL and can induce apoptosis of Fas expressing cells. They also harbor granules containing several proteases and the poreforming protein, perforin. Together, these proteins enter the target cell and initiate apoptotic signaling pathways. Perforin is believed to deliver granzyme B, a granule associated serine protease, into target cells. Granzyme B initiates apoptosis by activating the plasma membrane proximal protease, caspase 10. Granzyme B can also activate other caspases, presumably when other preferred substrates have been inactivated by viral inhibitors. Evidence suggests that Granzymes might also directly induce cell death independently of caspase activation (419).

In addition, Taniguchi and colleagues observed that infected cells can initiate a protective response for neighboring cells that involves IFN priming and IFN mediated destruction of infected cells (420). In this model, cells that are initially infected execute a signaling pathway that results in IFN release and self destruction. IFN induces apoptosis of virally infected cells while activating an antiviral mechanism that prevents infection of primed cells. This antiviral state can persist for days and even weeks.

5.1 Morphology of Apoptosis

Apoptosis is a programmed cell death that differs markedly from necrosis in appearance and in the mechanism of execution. Apoptosis involves a series of genetically controlled

steps and can be identified based on morphological changes that are induced in the dying cell. These cells are characterized by cytoplasmic blebbing, chromatin condensation and DNA fragmentation (222). Initial changes include exposure of phosphatidyl serine, normally present on the cytoplasmic face of the plasma membrane, to the extracellular leaflet of the lipid bilayer. Several host proteins are cleaved and chromatin condensation occurs. Chromatin condensation during apoptosis is not similar to the condensation of chromosomes seen in cell replication. Rather, apoptotic DNA condensation results from degradation of the nuclear lamina which under normal circumstances maintains nuclear structure (325). Several small nuclear bodies form due to the involution of the nucleoplasm and can be identified as circular blobs that stain brightly with DNA dyes such as Hoescht 33253 or propidium iodide. DNA is fragmented into nucleosomal multimers producing the classical DNA ladder. *In vitro*, the cell eventually ruptures into several membrane bound fragments referred to as apoptotic bodies; *in vivo* the cell is phagocytosed.

5.2 Molecular Components Regulating Apoptosis

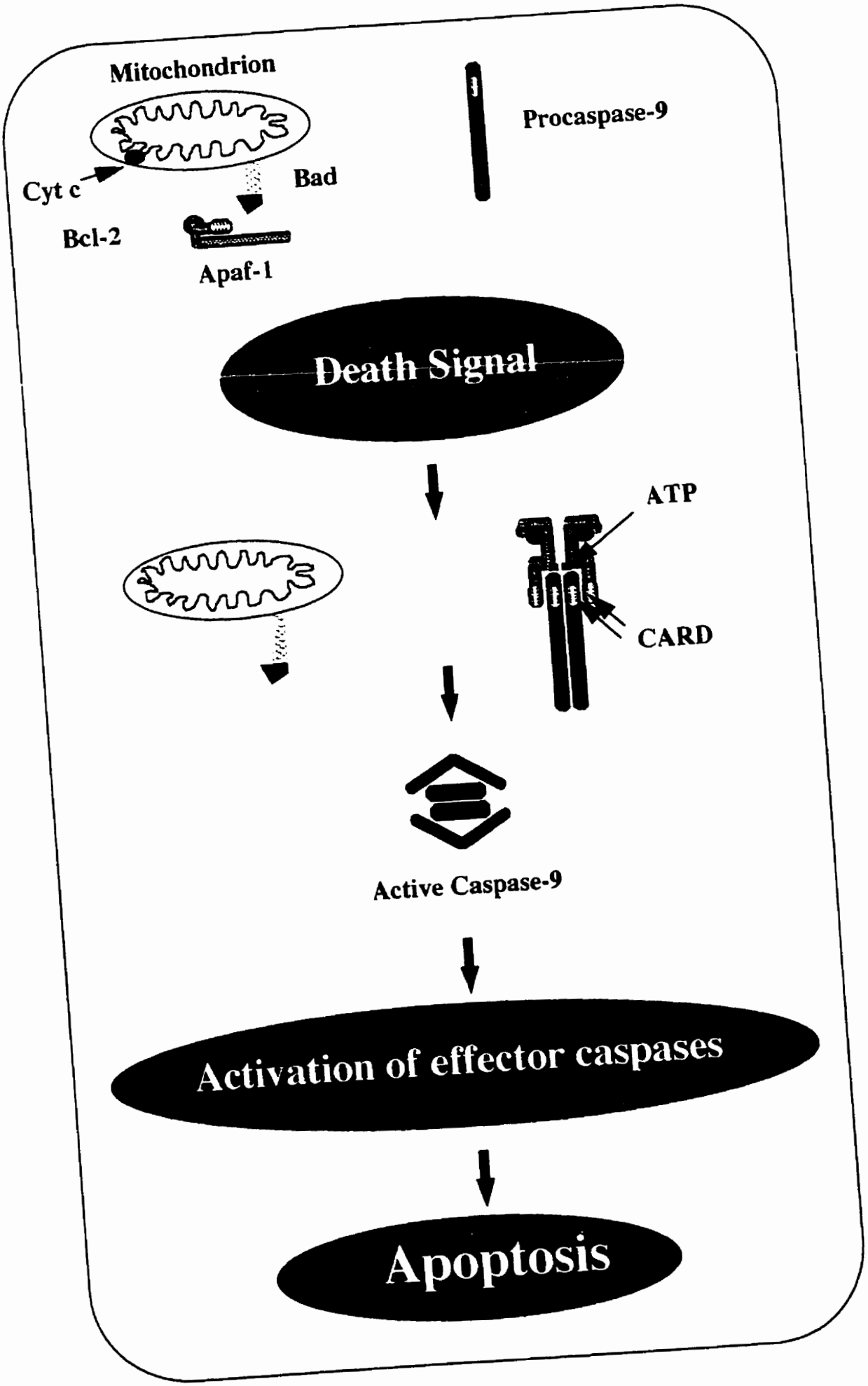
The molecular components regulating apoptosis are remarkably conserved throughout evolution although they exhibit greater complexity in mammalian systems. Developmental studies of the nematode *Caenorhabditis elegans* were instrumental in shedding light on the fascinating mechanism of signaling cell suicide. Genetic analysis found that three genes, CED-3, CED-4 and CED-9, are entirely responsible for the developmental programmed cell death seen in these creatures (185). CED-3 and CED-4 induce apoptosis while CED-9 is protective. Loss of function mutations in CED-3 and CED-4 prevent the death of nearly all cells usually programmed to die (128), while CED-9 loss of function mutations are lethal, presumably due to massive cell death (184). Further studies revealed that CED-9 functions to inhibit CED-4, which in turn activates CED-3. CED-4 interacts

with CED-3 and promotes its apoptotic inducing potential (299). CED-4 also interacts with CED-9 (77,463) and when all three proteins are expressed simultaneously, a trimolecular complex forms and apoptosis is prevented (393). This suggests that CED-9 blocks apoptotic signaling by interfering with the activity of CED-4 and CED-3.

Mammalian counterparts have been identified: CED-3 is a member of the caspase family of cysteine proteases, CED-4 is homologous to Apaf-1 and CED-9 is functionally and structurally similar to the Bcl-2 family of proteins. Numerous caspases have been identified, some of which are activated by Apaf-1 and others which act independently. The Bcl-2 family of proteins includes members that function similarly to CED-9 and protect cells from cell death, but also contains sub-families of pro-apoptotic members. A detailed description of these components is found below.

Recent studies have added further clarification to the mechanisms involved in signaling apoptosis. Apoptotic signaling can be initiated by a number of cellular insults including irradiation, incubation with chemotherapeutic drugs, the removal of environmental survival cues (352) or engagement of death receptor pathways (22). Two general mechanisms have been postulated, which can be executed singly or in concert depending on the death signal received. One pathway involves activation of initiator caspases which subsequently activates downstream caspases, bypassing the functions of Bcl-2 and Apaf-1. A second pathway involves the release of cytochrome c and apoptosis initiating factor (AIF) from mitochondria due to Bcl-2 family member initiated events, and the activation of caspases bound to Apaf-1. In this pathway, cytochrome c binds Apaf-1 in conjunction with ATP, allowing it to bind and activate procaspases which induces cell death. Bcl-2 anti-apoptotic members may prevent Apaf-1 from activating procaspases by binding and sequestering it, although this has not been formally shown (10). Figure 10 illustrates this theory. AIF release can also activate caspase 3, causing apoptotic cell death (414).

Figure 10. Theoretical model of mitochondrial control of apoptosis. Bcl-2 proteins are located on the cytoplasmic face of the mitochondrial membrane and may function to bind and sequester Apaf-1 preventing Apaf-1 activation of procaspase 9. Apoptotic signals might lead to the interaction of pro-apoptotic Bax with Bcl-2, causing the release of Apaf-1 and cytochrome c (cyt c). Cyt c and Apaf-1, in conjunction with ATP, would interact and activate procaspase 9, activating downstream effector caspases and culminating in cell apoptosis.


Mitochondrion
Cyt c
Bad
Bcl-2
Apaf-1
Procaspase-9
Death Signal
ATP
CARD
Active Caspase-9
Activation of effector caspases
Apoptosis

5.2.1 *Bcl-2 family of proteins*

The Bcl-2 family of proteins includes at least 15 mammalian members and several viral homologs (reviewed in 10). Each protein contains at least one of four conserved Bcl-2 homology domains (BH1 to BH4) and falls into two categories: proteins which protect cells from a variety of apoptotic stimuli such as Bcl-2 and Bcl-$_{xL}$ and those which are pro-apoptotic such as Bax and Bad. Anti-apoptotic members generally have at least BH1 and BH2 domains, and those most similar to Bcl-2 contain all four. The pro-apoptotic members can further be divided into two subfamilies which differ in their relatedness to Bcl-2. Bax, Bak and Bok contain BH1, BH2 and BH3 domains while others such as Bik, Blk and Bad possess only the central short (9-16 residues) BH3 domain. The BH3 domain proteins are unrelated to any other known proteins (81,221) and numerous studies have found that the BH3 domain is essential for the apoptotic function of these proteins.

Members can homo- and heterodimerize, and the relative ratio of different dimers may set the apoptotic threshold of a cell (323). Heterodimerization may be less important for pro-survival functions, since contradicting results have been observed (74,220), but appears essential for the pro-apoptotic function of BH3 domain proteins. Heterodimerization is important for Bax-like members but these proteins may have an additional cytotoxic function (described below).

Bcl-2 is found on the cytoplasmic face of the mitochondrial outer membrane, endoplasmic reticulum and nuclear envelope, although disruption of membrane docking does not necessarily affect its survival function (55,315). Only a fraction of Bcl-$_{xL}$ is found associated with membranes and Bax is cytoplasmic prior to an apoptotic stimulus, suggesting that Bcl-2 family members may associate with membranes by docking on certain organelle proteins (10).

Several theories have been proposed to explain Bcl-2 function. One theory suggests that Bcl-2 proteins may function by directly inhibiting the ability of Apaf-1 or related molecules to activate caspases (Figure 10). Bcl-$_{xL}$ can bind CED-4 and inhibit its activation by CED-3. Bcl-$_{xL}$ accomplishes this via its BH4 domain; this domain is required for its pro-survival activity and for interaction with CED-4 (200). Recently Bcl-$_{xL}$ has also been reported to interact with the CED-4 like portion of Apaf-1 and may inhibit the association of Apaf-1 with procaspase-9, preventing caspase activation. Pro-apoptotic family members such as Bik may antagonize Apaf-1 binding of anti-apoptotic Bcl-2 proteins, facilitating apoptosis (68,78).

Anti-apoptotic members may additionally prevent apoptosis by preserving organelle integrity. Bcl-2 can inhibit cytochrome c and AIF release from mitochondria. As mentioned earlier, cytochrome c with ATP, can induce Apaf-1 procaspase recruitment and activation (172,235,259,491) and subsequent cell death. However, it is unlikely that Bcl-2 functions solely to prevent cytochrome c release since Bcl-2 continues to protect cells after significant quantities of cytochrome c have been released(172). Hence whether organelle damage induces apoptosis or functions to amplify apoptotic signaling, remains to be resolved.

An alternative hypothesis suggests that Bcl-2 proteins may function by forming mitochondrial pores, and is based on the resemblance some Bcl-2 proteins share with poreforming bacterial toxins (309). Bcl-$_{xL}$, Bcl-2 and Bax can form ion channels *in vitro* (172) and this property may be important in regulating mitochondrial homeostasis. Bcl-2 and Bcl-$_{xL}$ can prevent the mitochondrial swelling and other alterations that occurs during apoptotic signaling, while Bax expression leads to the release of cytochrome c (281). Bax may therefore promote mitochondrial dysfunction, and heterodimerization with Bcl-2 or related members, may interfere with this function. Alternatively, Bax and Bcl-2 may

independently form ion channels that neutralize each other. In support of these mechanisms, Bcl-2 was recently shown to inhibit Bax ion channel activity (17).

In addition to promoting apoptosis by interacting with pro-survival family members, the Bax family of proteins may also kill by damaging organelles. Bax and Bak can kill yeast cells which do not express Bcl-2 like proteins or caspases (214), and cell death is abrogated by Bcl-2 co-expression due to preservation of mitochondrial integrity (172). Caspase independent death also occurs following overexpression of Bax like proteins (466). These proteins might hence cause cell death as a result of their channel forming abilities (491). Further studies are required however, to ensure that these results are not artifacts of overexpression.

5.2.2 *Caspases*

Caspases are a family of cysteine proteases (13 mammalian members and counting) which share similarities in amino acid sequence structure and substrate specificity (317). They are all expressed as pro-enzymes and range from 30-50-kDa. Caspases contain three domains: an N-terminal domain, a large subunit and a small subunit. Activation of caspases involves proteolytic processing between the three domains followed by association of the large and small subunit to form a heterodimer. Based on the crystal structures of some family members, active caspases are believed to exist as a tetramer with two catalytic sites that function independently.

Caspases are extremely specific proteases which recognize a tetrapeptide sequence and cleave after aspartic acid. The sequence recognized by different caspases is highly variable, explaining their broad range of biological activity (428). Not all proteins containing the optimal tetrapeptide sequence are cleaved, suggesting that structural

elements may be involved in substrate recognition. In addition, caspases are highly efficient with a k_{cat}/ K_m of greater than 10^6 M^{-1} s^{-1}.

Activation of caspases is believed to involve a complex proteolytic cascade system. These proteases can be divided into two groups: initiator caspases and effector caspases. Activation of initiator caspases requires binding of specific cofactors that are themselves activated by a pro-apoptotic signal. Binding is mediated by a structural motif that resides in both the caspase prodomain and its corresponding cofactor. Activation of procaspase 8 requires interaction with its cofactor FADD, and this interaction is carried out by the death effector domains (DED) (54). A second structural motif has been identified: caspase recruitment domains (CARD) mediate procaspase 9/Apaf-1 interactions (Figure 10) (258). Activation of procaspase 9 further requires cytochrome c and dATP indicating that multiple cofactors may be necessary for caspase activation. Activation of initiator caspases leads to the activation of effector caspases and progressive cellular disintegration. Caspase 3 has been identified as the major effector caspase in mammalian cells (316). Once activated, the apoptotic machinery is unidirectional and cannot be reversed.

Different initiator caspases mediate distinct signaling pathways. Caspase 8 is associated with death receptor induced apoptosis while caspase 9 is involved in death induced by cytotoxic agents. Both caspases activate effector caspase 3. This paradigm explains how different signals can lead to the induction of the same biochemical and morphological changes.

Numerous caspase substrates have been identified and for several, their cleavage contributes to the demise of the cell. Caspases can activate the nuclease responsible for DNA fragmentation, CAD (caspase-activated deoxyribonuclease), by cleaving its

associated inhibitor protein ICAD (131). Caspases can cleave Bcl-2 proteins which not only leads to their inactivation, but produces a fragment that induces apoptosis (467). Poly (ADP)-ribose polymerase (249), fodrin (100), p21 activated kinase 2 (PAK2) (379) and DNA fragmentation factor (DFF) (270) have also been identified as substrates. These proteases also directly cleave cell structural proteins such as nuclear lamins which causes the lamina to collapse and contributes to nuclear condensation (418). In addition, they inactivate proteins involved in DNA replication, DNA repair and mRNA splicing (99,358). Caspases hence induce apoptosis by attacking numerous signaling and structural elements, ensuring that the decided fate is executed without obstruction.

5.3 NF-κB Regulation of Apoptosis

Recently, NF-κB has been recognized as an important modulator of apoptotic cell death. In the vast majority of cases in which NF-κB is involved, it protects cells from apoptosis. NF-κB activation is protective against apoptosis induced by TNFα, ionizing radiation and some chemotherapeutic agents (32). Contrary to these situations, NF-κB sometimes functions to mediate apoptosis, although the mechanism involved remains to be elucidated.

The first suggestion that NF-κB was important in protecting cells from apoptosis came from RelA knock out mice (48). These mice die in utero and the liver cells of these mice undergo massive apoptosis. Fibroblasts and macrophages from RelA deficient mice are sensitized to TNFα and undergo rapid apoptosis compared to control cells (47); this phenotype can be reversed by transfection with RelA.

Several groups subsequently reported that cells expressing a dominant negative mutant form of IκBα are killed by TNFα and DNA damaging agents such as ionizing radiation

and the chemotherapeutic agent daunorubicin (47,437,444). Earlier studies had found that cells treated with TNFα or DNA damaging compounds (both treatments known to induce NF–κB), and protein or RNA synthesis inhibitors, underwent apoptosis. In light of the recent findings, it appeared that TNFα/synthesis inhibitor toxicity resulted from the inhibition of protective NF–κB regulated gene expression. Pretreatment with IL-1, a cytokine that induces NF–κB, prior to the addition of TNFα and synthesis inhibitor prevented apoptosis. Addition of IL-1 to cells already expressing a transdominant mutant of IκBα had no effect suggesting that IL-1 depended on NF–κB to mediate its protective function.

NF–κB also plays a role in protecting B cells from apoptosis. These cells express constitutively active NF–κB and inactivation by various means induces apoptosis (465). Constitutive NF–κB therefore ensures cell survival and hence cell proliferation. NF–κB may function to protect B cells from the enhanced levels of pro-apoptotic cytokines present in inflamed areas.

NF–κB protection from apoptosis is not always absolute and many cell types succumb to apoptosis upon prolonged stimulation even though NF-κB·DNA binding continues. A major function of NF–κB mediated apoptotic protection from compounds that induce both its activation and induce apoptosis may be to avoid unnecessary cell suicide until a certain response threshold is reached. This way, the organism can discriminate between cells that are irreversibly damaged or infected and cells that can be repaired.

As mentioned above, NF–κB can also mediate apoptosis. Glutamate induced toxicity of neurons is accompanied by NF–κB activation and can be inhibited by salicylate, an agent known to inhibit NF–κB (174). Apoptosis is also induced in a human embryonic kidney cell line upon serum withdrawal (176); transfection of a transdominant negative form of

Rel A, capable of suppressing κB dependent gene expression, partially reverses the apoptotic phenotype. NF–κB can activate genes involved in transducing apoptosis such as p53, c-Myc and Fas (32,205,217,464) and may mediate its apoptotic function through the transcriptional regulation of these molecules.

NF–κB as an inducer of apoptosis seems to be limited to a subset of cell types, although the nature of the apoptosis inducing stimulus may also be important. An essential role for NF–κB was recently identified in promoting double positive thymocyte apoptosis (189). Double positive thymocytes from transgenic mice expressing transdominant IκBα, mutated at serines 32 and 36, resisted anti-CD3 induced apoptosis through a pathway that involves Bcl-$_{xL}$. The thymocytes retained, however, normal sensitivity to γ-irradiation suggesting that NF–κB is dispensable for γ-irradiation induced apoptosis. NF–κB may accomplish these opposing functions by activating a distinct pattern of gene expression. Other cell type specific transcription factors may also be involved. Additionally, the effect of NF–κB may depend on the readiness of cells to undergo apoptosis and/or the number of apoptosis inducing versus protecting pathways affected by NF–κB.

5.4 Relevance of Apoptosis to AIDS Pathogenesis

The theory that apoptosis is a fundamental mechanism of HIV-1 induced CD4$^+$ T cell depletion and HIV pathogenesis is presently widely supported. Others have suggested that the decrease in CD4$^+$ T cells that results during HIV-1 infection is the result of lymphocyte retrafficking. Proponents of this theory suggest small changes in a subset's rate of exit, transit, or re-entry into tissues can have profound effects on the subset's relative ratio in blood (367). In addition, lymphocyte migration is profoundly affected by the inflammatory cytokines IFNγ and TNFα; upon injection of these compounds or

during infection, these cytokines can induce rapid depletion of lymphocytes from blood by increasing the rate at which lymphocytes exit the periphery and by reducing the rate at which they return (457). Indeed lymphocyte redistribution is expected to take place as a usual mechanism of T cell activation during viral infection, however studies have shown that considerable T cell apoptosis takes place in lymph nodes (144). Lymphocyte retrafficking into lymph nodes may actually serve to perpetuate HIV-1 induced apoptosis by recruiting uninfected cells to an area of high viral replication.

Numerous reports have suggested that HIV infection is associated with the induction of apoptosis in both infected and uninfected $CD4^+$ T cells (187 and references therein). *In vitro* infection of $CD4^+$ T cells by HIV-1 induces apoptosis (6,64) and *ex vivo* culture of T cells from asymptomatic HIV-infected individuals undergo apoptosis spontaneously (296). Further support is provided by the seminal studies from the Ho (195) and Wei (447) groups. These researchers found that a dramatic increase in $CD4^+$ T cell numbers occurs in many patients following treatment with potent antiretroviral drugs, implying a strong correlation between HIV replication and T cell depletion.

Several mechanisms have been proposed to account for the dramatic number of uninfected T cells undergoing apoptosis. One theory suggests that uninfected $CD4^+$ T cells undergo apoptosis due to engagement of CD4 molecules by Env protein present on neighboring HIV-infected cells and several studies have provided evidence for such a mechanism (80,142,395). Cross-linking of the CD4 molecule by injection of anti-CD4 antibodies leads to apoptosis of $CD4^+$ T cells, further supporting the plausibility of this mechanism (84). Additionally, CD4 engagement may signal the induction of molecules such as Fas and FasL (7), and several reports have implicated this pathway in HIV-1 induced T cell depletion (181).

Alternatively, a growing body of evidence suggests that HIV-1 infection of accessory cells such as monocyte derived macrophages play an important role in $CD4^+$ T cell apoptosis, particularly in uninfected T cells. Apoptosis of infected lymphocytes results largely from interaction with infected cells in the lymph nodes of infected individuals (144), and several studies have implicated macrophages as the main vehicle of viral transmission (26,27,172,257,454). This is particularly true after co-infection with opportunistic pathogens (324). Interestingly, induction of apoptosis as a result of CD4 cross-linking does not occur in purified peripheral blood lymphocytes (PBL) but only in unfractionated PBMC indicating that monocytic cells may be important for this method of cell killing as well.

Further supporting a role for monocytic cells in T cell depletion, are studies conducted using mouse models of HIV-1 infection. SCID mice reconstituted with adult human T cells and monocytes undergo more vigorous T cell depletion when *in vivo* infected with molecular clones that infect both monocytes and T cells, which are noncytopathic for $CD4^+$ T cell lines *in vitro*, than infection with molecular clones of HIV-1 that are highly cytophathic *in vitro* for $CD4^+$ T cell lines, but which do not infect monocytes (308). An elegant study by Herbein and colleagues used recombinant HIV-1 genomes encoding green fluorescent protein (GFP) to infect PBLs or mixed populations of PBLs and monocyte derived macrophages (187). Using flow cytometry to distinguish between GFP positive (and hence HIV positive) and negative cells, the authors found that HIV-1 infection of PBLs in the absence of macrophages resulted in the apoptotic death of predominantly GFP expressing cells. In contrast, HIV-1 infection of mixed populations resulted in a dramatic increase in apoptotic death of uninfected bystander cells with no increased apoptosis present in HIV-infected cells.

Recent studies also suggest that macrophages are necessary for the induction of apoptosis in $CD4^+$ T cells obtained from HIV-infected individuals but not for $CD4^+$ T cells obtained from uninfected people (26,27). Apoptosis was induced by TNFα and FasL in these cells and several other studies have documented the importance of these molecules in inducing apoptosis during HIV-1 infection (487). Together these results suggest that macrophages play a critical role in propagating cell death of uninfected $CD4^+$ T cells and hence the pathogenesis of HIV-1 infection.

Additionally, since macrophages are the scavengers of the immune system and avidly phagocytose cells undergoing apoptosis, viral DNA in apoptotic debris could transfect macrophages and lead to the production of new virions. Evidence for this theory comes from studies on the avian leukosis virus. This virus induces apoptosis, producing a classical DNA ladder. Southern blotting experiments revealed that linear unintegrated viral DNA survived this cleavage and was subsequently shown to generate a new infection albeit at a much lower rate than natural infection (452). Coupled with their long lived nature and their ability to travel virtually throughout the body, macrophages and cells of the myeloid lineage are likely to be critical mediators of viral spread and T cell death.

6.0 POTENTIAL INTERVENTIONS TO HIV-1 PATHOGENESIS

Tremendous advances in HIV-1 disease management have resulted due to the progress made in understanding the molecular biology and virology of HIV. Since the major factor in disease progression is depletion of functional $CD4^+$ T cells, it seems logical to suggest that therapies which interfere with $CD4^+$ T cell death would be beneficial. In fact, the opposite is true: inhibition of apoptosis in HIV-infected cells leads to enhanced virus production (16). Therapies which prevent HIV-1 replication and hence avert HIV-1 destruction of T cells, offer the best interventions to disease progression. It is well

accepted that destruction of the immune system begins during the earliest stages of HIV-1 infection and growing evidence suggests that inhibiting viral replication early on in disease offers the best hopes for disease management.

Numerous strategies that target different steps of the retroviral life cycle have been explored in the therapeutic treatment of HIV-1 disease. Two major classes of compounds have been approved: compounds that interfere with infection of target cells (i.e. nucleoside and non-nucleoside RT inhibitors) and those that suppress production of infectious virions in infected cell populations (i.e. protease inhibitors). Antiretroviral therapy for HIV infection has been shown to extend asymptomatic periods, reduce the frequency of opportunistic infections and improve survival rates (146,177,441). Clinical trials have found that combination therapy using agents that act at different steps of the HIV life cycle (i.e. RT inhibitors plus protease inhibitor) provide the greatest clinical benefit.

Nucleoside RT inhibitors (NRTI) were the first antiretroviral agents to be approved. These agents function as competitive inhibitors of the RT enzyme and lead to DNA strand termination during reverse transcription. Zidovudine or AZT, a thymidine analog, was the first RT enzyme to be approved both in the US and Canada. Clinical trials found that AZT delayed the onset of opportunistic infections, increased $CD4^+$ cell counts, and decreased mortality in symptomatic patients (145), but had a less demonstrable effect in asymptomatic patients with high $CD4^+$ cell counts (>500 cells/ml). AZT also has significant side effects which can be dose-limiting in the later stages of disease (381). Several other nucleoside inhibitors have since been developed including the purine analog dideoxyinosine (ddI), the cytosine analogs dideoxycytidine (zalcitibane or ddC), stavudine (d4T) and most recently lamivudine (3TC).

Prolonged NRTI monotherapy results in resistant viral strains (i.e. after 6 months) which can sometimes be circumvented or delayed using a combination of two NRTI. Interestingly, some AZT resistant HIV strains actually regain some AZT sensitivity in the presence of 3TC (246). 3TC may also restrict the selection of viral strains that are resistant to a number of other drugs (8), enhancing its efficacy in combination therapies.

The non-nucleoside RT inhibitors (NNRTI) are a second group of compounds that have been developed which inhibit RT by a unique, more specific mode of action than nucleoside analogs. This class includes unrelated compounds which demonstrate potent non-competitive inhibition of HIV RT *in vitro* with minimal cellular toxicity. Synergistic effects have been demonstrated *in vitro* with combinations of NNRTIs and nucleoside analogs. Two compounds have undergone extensive clinical study, nevirapine and delavirdine, and are presently available for use. These compounds are used primarily in combination with other NRTIs and protease inhibitors because of the potent effect of this combination and the rapid emergence of viral resistance to these agents individually (as early as 6 weeks) (310). The rapid emergence of resistant virus underscores the high rate of viral replication and $CD4^+$ T cell turnover that occurs at all stages of HIV infection (447).

Protease inhibitors have revolutionized HIV-1 disease treatment and have provided the greatest hope among the approved antiviral agents for long term therapeutic value. These compounds interfere with the viral aspartyl protease, necessary for the processing of polyprotein precursors into individual components, thus preventing formation of new infectious virus. Four protease inhibitors are presently available for use: saquinavir, indinavir, ritonavir and nelfinavir for use in combination with NRTIs, and several others are currently under development.

Triple combination therapies such as AZT/ddC/ritonavir or AZT/3TC/indinavir (HAART) result in a three log reduction in viral load in many patients, reducing viral levels in some cases to undetectable levels (303,343). Even used alone, protease inhibitors result in significant elevations in $CD4^+$ T cell numbers and decreased viral load (3,409). Although highly effective at reducing plasma viremia, these agents, are not without problems. Adverse effects and drug-drug interactions may be prohibitory, particularly in advanced patients taking numerous other medications. Suboptimal dosing also tends to select for resistant strains (66). Protease inhibitors are hence used only in combination therapy protocols.

Several other experimental protocols are under investigation. Hydroxyurea inhibits HIV-1 replication *in vitro* by depleting intracellular deoxynucleotide pools and studies have shown that non-toxic doses of hydroxyurea can synergise with nucleoside agents such as ddI (271). Bicyclams are also being investigated; these multiple-ring compounds inhibit virus uncoating and are potent *in vitro* inhibitors of HIV replication (8). Inhibitors which interfere with post-translational events and virion packaging are also presently being tested in clinical trials (353,359).

Although several inhibitors target the viral enzymes, RT and protease, no approved inhibitor exists for integrase. Several compounds which prevent HIV-1 integration are being developed; drug combinations targeting three viral enzymes could overwhelm the capacity of HIV-1 to tolerate mutations that confer drug resistance.

Tat and Rev inhibitors have been developed. Inhibition of Tat transactivation would significantly reduce viral replication, particularly bouts of replication that occur during co-infection with opportunistic infections. Inhibition of Rev function would halt viral production by blocking the translation of late stage viral proteins necessary for viral

replication. Tat antagonists (199) and Rev mutants (278) have been tested in clinical trials although only Rev mutants have shown therapeutic benefit.

In addition, gene therapy protocols have been developed by several researchers to inhibit HIV-1 replication. Antisense oligonucleotides, decoys of TAR and RRE and ribozymes have all been tested for their antiretroviral potential (268). Several of these protocols are in clinical trials (479), and although still largely experimental, these investigations will likely provide important insights into HIV pathogenesis.

Other strategies that have been employed include enhancement of HIV-1-specific immune responses using therapeutic vaccines (i.e. recombinant Env glycoproteins) (355), passive immunotherapy with monoclonal antibodies and *ex-vivo* expansion and reinfusion of autologous virus specific $CD8^+$ lymphocytes. Therapies aimed at inhibition of cytokines which induce HIV-1 replication such as TNFα are also being studied (117,276).

The greatest hope for global HIV management resides in vaccine development. The cost of antiretroviral treatment for one individual can reach upwards of $20 000/year. Since, the majority HIV infected individuals live in developing countries, antiretroviral therapies are not an available option. Vaccine development is problematic for several reasons. HIV-1 includes numerous virus subtypes which are categorized into clades and each clade comprises additional subtypes. In addition, the high mutational capacity of HIV-1 Env proteins and their apparent lack of immunogenicity make them unlikely candidates for a protective vaccine. An international effort to produce an HIV vaccine has made significant progress in a relatively short time, in terms of vaccine development. Continuing efforts will hopefully make this possibility a reality: the fate of developing countries where HIV has devastated entire communities, may depend on it.

Specific Research Aims

The primary objective of the research presented in this thesis was to elucidate the molecular mechanisms leading to constitutive NF-κB activation in HIV-infected myeloid cells and to examine the nature of this activity. To accomplish this, the work has been divided into three specific research aims:

1) To examine the activation of PKR and assess its involvement in modulating the NF-κB/IκB pathway in HIV-infected myeloid cells.

2) To study the contribution of IκBβ to constitutive NF-κB activation and decipher its participation in maintaining transcriptionally active NF-κB.

3) To investigate the function of NF-κB activation in modulating apoptotic pathways in HIV-infected cells.

This work characterizes signaling pathways that are deregulated in HIV-1 infected myeloid cells and analyses their contribution to constitutive NF-κB activation. The experiments performed focus on the inhibitory proteins, IκBα and IκBβ, and examine their individual contribution to NF-κB activation using two models of chronically HIV-infected myeloid cells. This work further investigates the effect of constitutive NF-κB activation on apoptotic signaling in HIV-1 infected myeloid cells, and investigates the possible mechanisms involved. Based on these results, we propose a model in which HIV-1 induces NF-κB activation by modulating upstream signaling pathways to transcriptionally induce anti-apoptotic genes important for cell viability.

CHAPTER II

MATERIALS AND METHODS

Cell culture. Promonocytic U937 and HIV-infected U9-IIIB cells, as well as myelomonoblastic PLB-985 and HIV-infected PLB-IIIB cells (infected with HIV strain IIIB) were maintained in RPMI 1640 (GIBCO, Life Technologies Inc.) supplemented with 5% Fetal Clone (Hyclone), 2 mM L-glutamine, and 20 μg/ml gentamicin (Schering Canada). Jurkat T cells stably expressing doxycycline (Dox) inducible transdominant mutants of IκBα - (rtTA Jurkat 2N, containing an IκBα mutated in at S32A/S36A; rtTA Jurkat 2NΔ4, containing an IκBα mutated at S32A/S36A and 22aa C-terminal truncation - or the empty Neo vector rtTA-Neo-Jurkat) were previously described (239). Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum 2 mM L-glutamine, and 20 μg/ml gentamicin. The transgenes were induced by the addition of Dox (1 μg/ml) to the culture medium for a minimum of 16h. Cells were seeded at a density of 0.5 x 10^6 cells/ml for all experiments and treated with various inducers as indicated in the figure legends: 10-30 ng/ml of recombinant human tumor necrosis factor α (TNFα; 0.1% BSA in phosphate buffered saline (PBS; R&D Systems), 5-10 ng/ml IL-1β (0.1% BSA in PBS; R&D Systems), 50 ng/ml phorbol myristate acetate (PMA in EtOH; ICN) 30-50 mM N-acetyl-cysteine (NAC, in PBS, pH 7.5; Boehringer Mannheim) and 50-100 μg/ml cycloheximide (CHX; Sigma).

Reverse transcriptase analysis. Cell culture supernatants were clarified by centrifugation at 3000 RPM for 30 min. A 50 μl aliquot of the supernatant was incubated in 50 mM Tris-HCL pH 7.9, 5 mM $MgCl_2$, 150 mM KCl, 0.05% Triton X-100, 0.3 mM glutathione (reduced) 0.5 mM [Ethylenebis(oxyethylenenitrilo)]-tetraacetic acid (EGTA). 50 μg/ml poly rAdT (Pharmacia), and 10 mCi tritiated thymidine triphoshhate (^{3}H-TTP; ICN Biomedicals) for 22 h at 30°C. Reverse transcription was stopped by adding 2 ml of 0.01M sodium pyrophosphate in 1N HCl, 2 ml of ice cold 10% trichloroacetic acid (TCA) and placing the tubes on ice. The resulting precipitates were then collected on Whatman

GF/C filters and washed several times with cold 5% TCA and once with 70% ethanol. Filters were air dried, then counted by liquid scintillation fluorography.

Antibodies. NF-κB antisera were prepared as described previously (335). Antibody AR27 or anti-p105-N was raised against a peptide (amino acids 2-15) at the most N-terminal region of p105, and recognizes both p105 and p50; AR43 or anti-p100-N, was generated against a peptide at the N-terminus of p100 (amino acids 2-17), and recognizes both p100 and p52; AR28 recognizes the C-terminus of RelA (amino acids 537-550); AR22 recognizes the C-terminus of c-Rel (amino acid 573-587). AR20 recognizes the N-terminus of IκBα (aa2-16). Antiserum that recognizes the N-terminus of RelA was purchased from Santa Cruz Inc. IκBβ antibodies C20 (recognizes 43-kDa isoform) and G20 (recognizes both 43- and 41-kDa forms) were purchased from Santa Cruz. The phosphoserine 32 IκBα antibody was purchased from NEB and the monoclonal IκBα antisera MAD-10B, was a kind gift from Ron Hay (208). The alpha-tubulin antibody was obtained from ICN and the actin antisera from Sigma. Monoclonal anti-Bcl-2 and polyclonal anti-Bax were purchased from Santa Cruz Inc.

Total RNA isolation. Total cellular RNA was isolated from cells using a modified quanidium isothiocyanate procedure (83). Cells were pelleted by centrifugation, and then resuspended in 500 µl of Solution D (4 M guanidium isothiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, and 100 mM β-mercaptoethanol). 500 µl of water saturated phenol, 50 µl of 0.5 M sodium acetate, pH 4.0 and 1 ml of chloroform: isoamyl alcohol (49:1) were added and vortexed. The mixture was centrifuged at 10 000 RPM for 20 min. The upper aqueous layer was removed and nucleic acids precipitated with an equal volume of isopropanol. The mixture was centrifuged at 10 000 RPM for 20 min and the pellet resuspended in dH_20. The RNA was then treated with RNAse-free DNAse (RQ1 DNAse;

Promega) for 30 min at 37°C in 40 mM Tris-HCl pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$. The RNA was phenol extracted, ethanol precipitated and stored at -80°C.

S1 nuclease analysis. Total cellular RNA from PLB-985 and PLB-IIIB cells was isolated at specific time points after infection according to a modified guanidium isothiocyanate procedure. S1 mapping analysis was performed using 40 μg of RNA and 5' [γ-^{32}P] ATP end-labelled probes specific for IFN-α1, and IFN-β as previously described (193). Nuclease resistant DNA/RNA hybrid molecules were resolved on a 6% denaturing polyacrylamide gel, quantified by laser densitometry and expressed as relative RNA levels.

Assay for PKR activity. PLB-985 and PLB-IIIB cells were concentrated to $1x10^7$ cells/ml and incubated with Sendai virus (100 HAU/ml) for 90 min at 37°C. Mock infected cells were concentrated as described but incubated without virus. Cells were subsequently diluted to $1x10^6$ cells/ml and incubated for a total of 6h. Cells were then lysed with lysis buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM DTT, 2 mM $MgCl_2$, 1% Triton X-100, 0.2 mM PMSF, 3 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μ/ml pepstatin). Extracts (100 μg) were incubated with anti-PKR antibody for 2h at 4°C. Anti-mouse IgG agarose beads were added to the extracts and incubated for an additional 2h at 4°C. Beads were washed four times with high salt buffer (20 mM Tris-HCl, pH7.5, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 1% Triton X-100, 0.2 mM PMSF, 3 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 20% gycerol) followed by three washes with low salt buffer (10 mM Tris-HCl, pH7.5, 100 mM KCl, 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 3 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 20% glycerol). PKR activity was induced by incubating the beads with 0.1 μg/ml poly rI:poly rC (Pharmacia) in 5X kinase buffer (50 mM Tris-HCl pH 7.7, 250 mM KCl, 10 mM MgCl2, and 25 mM ß-mercaptoethanol) for 10 min on ice. The beads were then incubated at 30°C for 30 min with [γ-^{32}P] ATP (ICN) to detect autophosphorylation. PKR was solubilized with 2X SDS

sample buffer and run on an 8% polyacrylamide gel. The gel was fixed with 10% methanol, 5% acetic acid for 30 min, dried and exposed to X-ray film. The intensities of the PKR bands detected by autoradiography were quantified by laser densitometry (Pharmacia).

Northern blot analysis. Total RNA (10-20 μg) was electrophoresed in a 1% denaturing formaldehyde agarose gel and transferred to a nylon membrane (Hybond-N; Amersham) with a Vacu-Gene blotting system (Pharmacia). RNA was cross-linked to the membrane in a UV Stratalinker 2400 (Stratagene) and prehybridized overnight in Prehybridization solution: 25 mM KPO_4 pH 7.4, 5X SSC (750 mM NaCl, 75 mM sodium citrate pH 7.0), 50% deionized formamide, 50 μg/ml salmon sperm DNA (Boehringer Mannheim) and 5X Denhardt's solution containing 0.5% (w/v) Ficoll 400 (Sigma), 0.5% (w/v) polyvinylpyrrolidine, and 0.5% (w/v) BSA at 42°C in a hybridization oven. **Probe preparation:** The probes include: a 0.8 kb PKR fragment generated by EcoRI digestion of plasmid p68wt-pcDNAI/NEO (232), a 1.1 kb fragment isolated from pSVK3-IκBα by EcoRI digestion (43) and 1.1 kb fragment derived from pβ–actin by PstI digestion. Probes were labeled to approx. 1 x 10^9 cpm/μg with the Oligolabeling kit (Pharmacia) and [α–^{32}P]CTP. Hybridization occurred overnight at 42-50°C in Prehybridization solution supplemented with 10% (w/v) dextran sulfate (Pharmacia) and approximately 50 million cpm of labeled probe. The blots were washed, wrapped in plastic and exposed to X-OMAT film (Kodak) at -80°C.

Western blot analysis. Whole cell extracts were prepared by resuspending in NP-40 lysis buffer (10 mM Tris-HCl pH 8.0, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 0.5% NP-40, 0.5 mM PMSF, and 0.01 mg/ml each of leupeptin, pepstatin and aprotinin). After incubation on ice for 10 min, cellular debris was removed by centrifugation for 10 min at 4°C. Cell extracts (10-50 μg) were resolved by SDS-PAGE and then transferred to a

nitrocellulose membrane. Membranes were blocked for a minimum of 2h in 5% skim milk and incubated overnight in 5% milk containing anti-peptide monoclonal or polyclonal antisera (dilutions ranged from 1:250 to 1: 1000). The membranes were rinsed four times in PBS and incubated with a secondary antibody, HRP-conjugated goat anti-rabbit or rabbit anti-mouse (1:1000, Amersham) for 1 hr at room temperature and then rinsed again four times in PBS. The ECL-western blotting detection system (Amersham) was used according to manufacturer's instructions to visualize the specific signals. Autoradiograms in chapter III were scanned by laser densitometry and the intensity of each band is presented relative to levels in uninfected, unstimulated cells for NF-κB subunit blots and in terms of % IκBα remaining relative to time zero for IκBα turnover blots. Autoradiograms in chapter V were scanned using a laser scanner and quantified using NIH Image 1.60 software. Bcl-2:actin ratios are presented relative to levels in unstimulated cells. Values were plotted on a semi-log scale and the best fit curves were determined. The experiments were repeated a minimum of three times and representative autoradiograms are shown.

Detection of IκBα Phosphorylation. Cell extracts were prepared as for western analysis and were left untreated, treated with 0.5 units of potato acid phosphatase in PIPES buffer (10 mM PIPES pH 6.0, 0.5 mM PMSF, 5 μg/ml of each leupeptin, pepstatin and aprotinin), or treated with 0.5 units or potato acid phosphatase in PIPES buffer plus inhibitor mix (10 mM NaF, 1.5 mM Na_2MoO_4, 0.4 mM Na_3VO_4 and 0.1 μg/ml okadaic acid) at 37°C for 60 min. Equivalent amounts of protein (50 μg) were resolved using a 5-13% gradient SDS polyacrylamide gel and were transferred to nitrocellulose. IκBα and phosphorylated IκBα were visualized using the previously described affinity purified IκBα antibody (AR20). In other experiments, PLB-985 and PLB-IIIB cells in log phase growth were concentrated to 1.0 x 10^6 cells/ml and were incubated with 25 μM MG132 (Myogenics) or with an equal volume of DMSO for 0-120 min, as indicated. Cell extracts were electrophoresed in a 5-13% SDS polyacrylamide gradient gel and transferred to

nitrocellulose for immunoblot analysis. Phosphorylated IκBα was detected as a distinct slower migrating band using a monoclonal IκBα peptide antibody (363).

GST fusion proteins. IκBα human cDNA bearing a 22 amino acid C-terminal truncation in the PEST domain (IκB-Δ4, inhibits NF-κB binding as efficiently as wild type) was subcloned into pGEX 2T as previously described (264). Wild type GST-IκBα (1-55) and mutant GST-IκBα (1-55; S32/36A) expressing plasmids, subcloned in pGEX 2T, were received as a gift from Antonis Koromilas. DH5α bacteria expressing GST-IκB proteins were grown in Luria broth overnight at 30°C with shaking and induced with 1 mM isopropylthio-β-D-galactoside (IPTG; Pharmacia) for 3h the following morning. Cells were washed with PBS, resuspended in 10% Triton in PBS and sonicated. Protein was recovered using Sepharose 4B glutathione beads (Pharmacia Biotech) and eluted with 20 mM GSH (Calbiochem) in 50 mM Tris pH 8.0. Isolation purity and quantity were confirmed by SDS-PAGE Coomassie blue staining and the visual comparison to BSA standards.

Nuclear and cytoplasmic extracts. Cells were harvested by centrifugation and washed once with ice cold PBS and once with Buffer A (10 mM HEPES pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl; 0.5 mM dithiothreitol (DTT) and 0.5 mM phenyl-methyl-sulfonyl-fluoride (PMSF)). Pellets were resuspended in Buffer A containing 0.1% NP-40 and chilled on ice for 10 min before being centrifuged at 14 000 RPM. Cytoplasmic fractions were removed and frozen at -80°C and pellets were resuspended in Buffer B (20 mM HEPES pH 7.9; 25% glycerol; 0.42 M $NaCl_2$; 1.5 mM $MgCl_2$; 0.2 mM EDTA; 0.5 mM DTT; 0.5 mM PMSF; 5 μg/ml leupeptin; 5 μg/ml pepstatin; 0.5 mM spermidine; 0.15 mM spermine and 5 μg/ml aprotinin). Samples were incubated on ice for 15 min before being centrifuged at 14 000 RPM for 10 min. Nuclear extract supernatants were diluted with

Buffer C (20 mM HEPES pH 7.9; 20% glycerol; 0.2 mM EDTA; 50 mM KCl; 0.5 mM DTT and 0.5 mM PMSF), snap frozen using dry ice and stored at -80°C.

Electromobility shift assay. Nuclear extracts were preincubated with 5 μg of the non-specific DNA competitor poly (dI:dC; Pharmacia) for 10 min in a total volume of 15-20 μl of DNA binding assay buffer (20 mM HEPES pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA. 0.2 mM,EGTA 2 mM DTT, 0.1% NP-40). Binding activity was assessed using 0.2 ng of an NF-κB ^{32}P-end-labeled probe corresponding to the PRDII region of the IFN-β promoter (5'-GGAAATTCCGGGAAATTCC-3') incubated with the extract for 20 min. Protein-DNA complexes were then separated on a 5% native polyacrylamide gel (60:1 cross-link) in Tris-glycine (25 mM Tris, 195 mM glycine, pH 8.5). In supershift experiments, antibody and its corresponding peptide where indicated (Santa Cruz), were incubated with 5 μg of nuclear extract for 20 min. Poly dI:dC (5μg) was added for an additional 10 min followed by incubation with labeled probe for 20 min. All steps were carried out at room temperature. In other experiments where GST fusion proteins were used, nuclear extracts were incubated with poly dI:dC for 10 min and then incubated with labeled probe for 20 min. Increasing amounts of GST-IκBα Δ4 (10 ng/μl) was added for an additional 20 min. The resulting protein DNA complexes were resolved by a 5% tris-glycine gel and exposed to X-ray film. To demonstrate the specificity of protein DNA complex formation, 125 fold molar excess of unlabeled oligonucleotide was added to the nuclear extract before adding the labeled probe.

IκBβ immunodepletion. Cells were harvested by centrifugation and washed with ice cold PBS. Pellets were resuspended in TNN buffer (20 mM Tris pH 7.5; 200 mM NaCl; 0.5% NP-40, 0.5 mM PMSF, 5 μg/ml of each aprotinin, leupeptin and pepstatin; 0.5 mM spermine; 0.15 mM spermidine; 1 mM NaF; 1 mM $NaVO_4$) and incubated on ice for 10 min. Supernatants were removed after centrifugation (14000 RPM, 10 min, 4°C) and

assayed for protein concentration. 200 μg of protein was diluted to 400 μl with TNN and incubated with 10 μl of IκBβ C-20 antibody for 2h at 4°C with rotation. 30 μl of washed protein A conjugated Sepharose beads were added and the mixture rotated at 4°C for an additional hour. Samples were centrifuged and supernatants assayed by EMSA or western blot.

Transfections and CAT assays. Human embryonic kidney 293 cells were transfected in 100 mm plates by the calcium phosphate DNA precipitate transfection method (1). Each plate was transfected with 7 μg of NF-κB CAT (PRDII element of the IFNβ gene linked to CAT; described in 192) and either: 4 μg of pSVK3- IκBα (described in 14) and 4 μg pSVK3 empty vector, with 4 μg of pSVK3-IκBα and 4 μg pSVK3-IκBβ (described in 14) or 8 μg empty vector. DNA was resuspended in 450 μl of dH_20 and combined with 50 μl of 2.5 M $CaCl_2$. The DNA/$CaCl_2$ mixture was added dropwise to 500 μl of 2X HEPES buffered saline (0.05 M HEPES, 0.28 M NaCl, 1.5 mM Na_2HPO_4, pH 7.05) and incubated for 20 min at room termperature to form a preciptiate. Cells were incubated with precipitate for 12h after which they were washed with PBS and resuspended in fresh media. Cells were maintained for an additional 36h and stimulated with PMA (50 ng/ml) for the last 24, 16 or 8h of the transfection. CAT activity was determined using 100 μg of total cell extract assayed for 2h at 37°C according to Gorman et. al. (169). Quantitation of the percent acetylation was performed using NIH Image 1.60. The fold activation reported is the average of a minimum of 3 experiments with error bars representing the standard deviation.

IKK assay. Cells were pelleted by centrifugation and washed with ice cold PBS. Pellets were resuspended in TNN buffer (20 mM Tris pH 7.5; 200 mM NaCl; 0.5% NP-40, 0.5 mM PMSF, 5 μg/ml of each aprotinin, leupeptin and pepstatin; 0.5 mM spermine; 0.15 mM spermidine; 1 mM NaF; 1 mM $NaVO_4$) and incubated on ice for 10 min. Supernatants

were removed after centrifugation (14000 RPM, 10 min, 4°C) and assayed for protein concentration. 150-300 μg of protein extract was incubated with 5 μl IKKα antibody (Santa Cruz) or 2 μl of normal rabbit serum for 2h rotating at 4°C. 20 μl of protein A conjugated Sepharose beads, washed 3X with TNN buffer, was added to the mixture and incubated with rotation at 4°C for an additional hour. Beads were pelleted (1000 RPM) and washed once with TNN and 2X with kinase buffer (50 mM Tris pH 8.0; 100 mM NaCl; 2 mM $MgCl_2$; 0.5 mM PMSF, 4μg/ml chymostatin; 5 μg/ml of each pepstatin, leupeptin and aprotinin, 1 mM of each DTT, NaF and $NaVO_4$). Beads were resuspended in 20 μl of kinase mix containing 2 μg GST-IκBα (1-55) or IκBα (1-55; S32/36A) and 0.5 μCi γ-ATP and incubated for 30 min at 30°C. 20 μl of 2X SDS loading dye was added to each reaction, samples were boiled for 5 min and separated on a 10% SDS polyacrylamide gel. The gel was fixed and Coomassie stained (5% acetic acid, 10% ethanol, Coomassie blue dye), subsequently destained (5% acetic acid, 10% ethanol), dried and exposed to film for 1-3h at -80°C.

DNA Fragmentation. Following treatments, ~2 x 10^6 cells were pelleted, washed with PBS, resuspended in 250 μl of lysis buffer (20 mM Tris HCl pH 7.5; 10 mM borate; 0.25% NP-40; 0.1 mg/ml RNase) and incubated for 1h at 37°C. Proteinase K was added to a final concentration of 1 mg/ml and extracts were incubated for an additional hour. Samples were separated on a 1.8% agarose gel containing 0.5 μg/ml ethidium bromide and visualized by UV illumination.

TUNEL Analysis Apoptosis was quantified using an *in situ* cell death detection kit (Boehringer Mannheim). Approximately 1 x 10^6 cells were centrifuged, washed once with PBS and resuspended in 20 μl of PBS. Cells were plated on a multichamber slide, air dried and fixed with 4% paraformaldehyde for 30 min at room temperature. Slides were rinsed twice with PBS and incubated for 2 min at 4°C in permeabilisation solution (0.1% Triton;

0.1% sodium citrate), rinsed with PBS and incubated with fluorescein labelled TUNEL reaction mixture for 1h at 37°C in a humid, darkened chamber. Slides were again rinsed with PBS, incubated with the nuclear dye HOESCHT 33342 (0.4 ng/ml) to stain all nuclei, washed with PBS and embedded in mounting solution (10 mM Tris HCL, pH 8.8; 0.1M propyl gallate in glycerol). Samples were analysed by fluorescence microscopy and the percentage of apoptotic cells was determined by counting a minimum of 350 nuclei (blue filter) and the corresponding TUNEL positive cells (green filter).

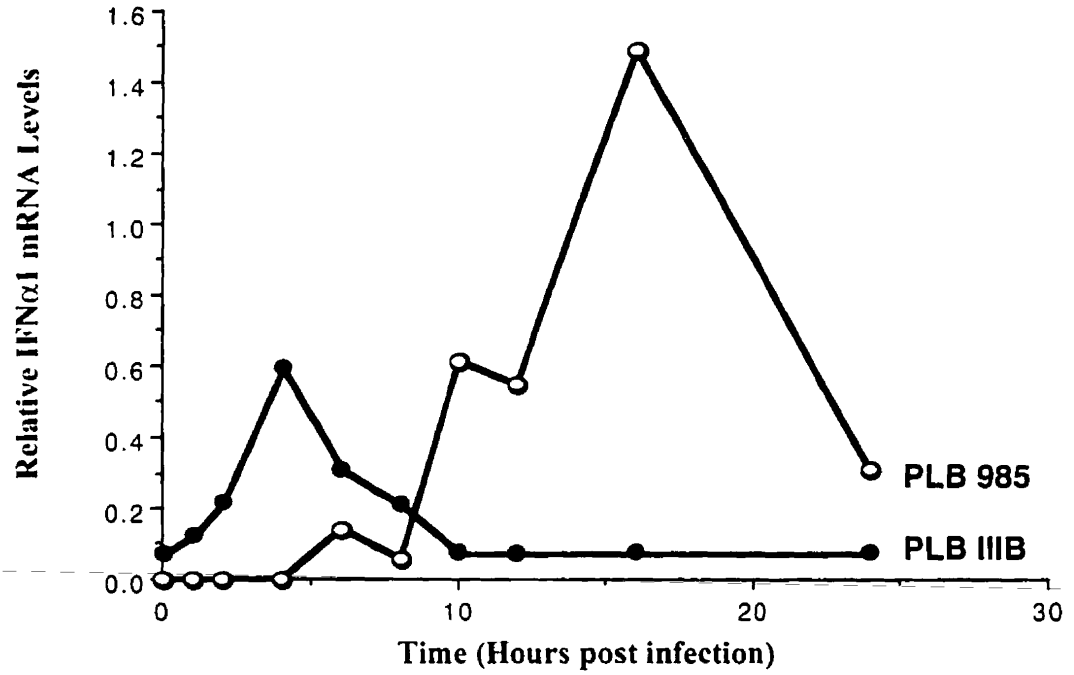
CHAPTER III

CHRONIC HIV-1 INFECTION OF MYELOID CELLS DISRUPTS THE AUTOREGULATORY CONTROL OF THE NF-κB/REL PATHWAY VIA ENHANCED IκBα DEGRADATION

U937 and PLB-985 cells are $CD4^+$ monocytic cell lines. U937 are promonocytic cells which originated from a patient with histiocytic lymphoma (413); PLB985 are characterized as a diploid myelomonoblastic cells derived from a patient with acute non-lymphocytic leukemia (435). Chronically infected cells were generated *in vitro* using the HTLV-IIIB strain of HIV-1 and phenotypically characterized (372). The maintenance of chronic HIV-1 infection was monitored by measuring RT activity as described in materials and methods. HIV-1 infection induces monocytic differentiation in these cells and leads to the constitutive activation of NF-κB DNA binding (370,372). These cell lines were used as a model of myeloid HIV infection.

Altered kinetics of IFN gene expression. Since HIV infection leads to the dysregulation of numerous cytokine genes (373), we sought to examine whether IFN production was affected. A kinetic analysis of IFNα1 and IFNβ gene transcription was performed in PLB-985 and PLB-IIIB cells. S1 mapping analysis using probes corresponding to IFNβ and IFNα1 demonstrated that chronically infected PLB-IIIB cells constitutively expressed a low level of IFNα1 and IFNβ mRNAs (371,372). When co-infected with Sendai virus, PLB-IIIB cells exhibited an altered pattern of IFN gene expression compared to Sendai virus infection of PLB-985 cells (Fig. 11). Sendai-induced IFN mRNA from PLB-IIIB cells increased at 1h post infection (p.i.), reached a peak at 4-6h, and then decreased to constitutive levels by 10h p.i. In contrast, Sendai-induced IFN mRNA from PLB 985 cells was initially detected at 6h p.i. and reached a peak at 10-16h (Fig. 11). This experiment demonstrated three distinct alterations in IFN induction: 1) a constitutive level of transcription in chronically HIV-infected PLB-IIIB cells; 2) a rapid induction kinetics in Sendai virus infected PLB-IIIB compared to PLB-985 cells; and 3) a lower relative amount of IFN mRNA in Sendai induced PLB-IIIB cells compared to PLB-985 cells.

Figure 11. Kinetics of type 1 IFN mRNA expression in PLB-985 cells and PLB-IIIB cells after infection with Sendai paramyxovirus. Total cellular RNA was extracted from PLB-985 and PLB-IIIB cells at hours 0, 1, 2, 4, 6, 8, 10, 12, 16, and 24 following Sendai virus infection. S1 mapping was performed using 40μg of cellular mRNA and an [γ-^{32}P]ATP end labeled IFNα1 and IFNβ specific probes, as previously described (193, 372); signals corresponding to RNA-DNA hybrids were identified by autoradiography and the intensity of the bands corresponding to the IFNα1 and IFNβ signals were scanned by laser densitometry and plotted in terms of relative mRNA levels. The kinetics of IFNα1 are illustrated and are qualitatively similar to results obtained for IFNβ.


Relative IFNα1 mRNA Levels
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
Time (Hours post infection)
PLB 985
PLB IIIB

Analysis of PKR expression and activity. PKR is induced by IFN and is activated in the presence of natural or synthetic dsRNA. Since recent studies demonstrated that PKR activated NF-κB DNA-binding activity in cellular extracts (236,282), we sought to examine whether PKR activity may be elevated in HIV-infected cells and play a role in IκBα phosphorylation and turnover. Northern blot analysis demonstrated a constitutive level of PKR mRNA expression in PLB-IIIB cells (Fig. 12A, lanes 4-6) compared to PLB-985 cells (Fig. 12A, lanes 1-3). Also, the expression of PKR mRNA in PLB-IIIB cells increased in response to PMA for 8h (Fig. 12A, lane 5) but not in response to Sendai virus infection (Fig. 12A, lane 6). In other experiments, PKR mRNA levels peaked at 24h p.i. in PLB-985 cells, while in PLB-IIIB PKR mRNA levels were constitutively elevated (data not shown). Similarly, the level of PKR protein was increased by more than five-fold in PLB-IIIB cells compared to PLB-985 (Fig. 12B). Finally, analysis of PKR autocatalytic activity demonstrated that PKR activity was increased in extracts from PLB-IIIB cells compared to PLB-985 cells (Fig. 12C). Longer exposures revealed that PKR activity was detectable in PLB-985 cell extracts stimulated by Sendai infection (data not shown). These results demonstrate that HIV infected myeloid cells have increased PKR activity compared to similarly treated PLB-985 cells.

Addition of neutralizing IFN antibody downregulates PKR levels. Kinetic analysis revealed that PKR protein levels were inducible in PLB-985 but were essentially constitutive in PLB-IIIB cells (Fig. 12 and 13A). Proteins levels were increased in PLB-985 cells at 18-24h p.i. (Fig. 13A, lane 5) but only weakly elevated at 6h. p.i. (Fig. 13A, lanes 2-4). In contrast, PLB-IIIB cells had high PKR levels at various time points between 0 and 48h after treatment with different activators (Fig. 13A, lanes 6-10 and Fig. 13B, lanes 5-9, 11,12). Since IFN expression may lead to elevated levels of PKR in chronically infected cells, abrogating IFN signaling with the addition of a neutralizing antibody may decrease PKR expression. To determine whether anti-IFN α/β antibody would affect PKR

Figure 12. Analysis of PKR mRNA and protein expression levels in PLB-985 and PLB-IIIB cells. Total cellular RNA was isolated from PLB-985 (lanes 1-3) or PLB-IIIB (lanes 4-6) cells that were untreated (U), PMA treated (P), or Sendai virus infected (S) for 8h. (**A**) RNA was separated on a 1% denaturing agarose gel containing ethidium bromide, and visualized under UV light (310 nm). After RNA was transferred to a nylon membrane, the blot was probed with a PKR cDNA probe. The position of the 2.5 kb PKR RNA mRNA is indicated by an arrow. (**B**) PLB-985 cells (lanes 1 and 2) or PLB-IIIB cells (lanes 3 and 4) that were untreated (U) or infected by Sendai virus for 6h were separated by SDS-PAGE and then transferred to a nitrocellulose membrane and immunoblotted with a monoclonal PKR antibody. The arrow indicates the band corresponding to the 68 kDa PKR protein. (**C**) PLB-985 cells (lanes 1 and 2) or PLB-IIIB cells (lanes 3 and 4), untreated (U) or infected by Sendai virus for 6h (S), were immunoprecipitated with a polyclonal antisera against PKR. PKR activity was induced by poly rI:poly rC and incubated with [γ-^{32}P]ATP to detect autophosphorylation. Signal was visualized by autoradiography.

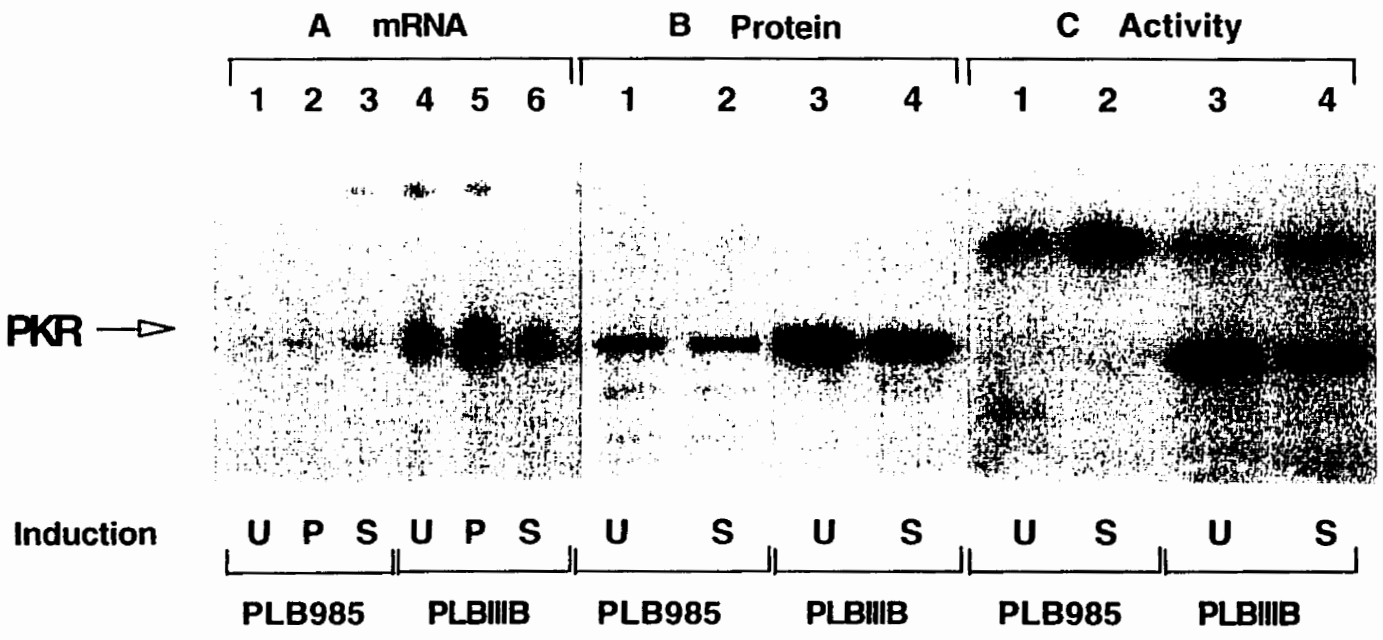
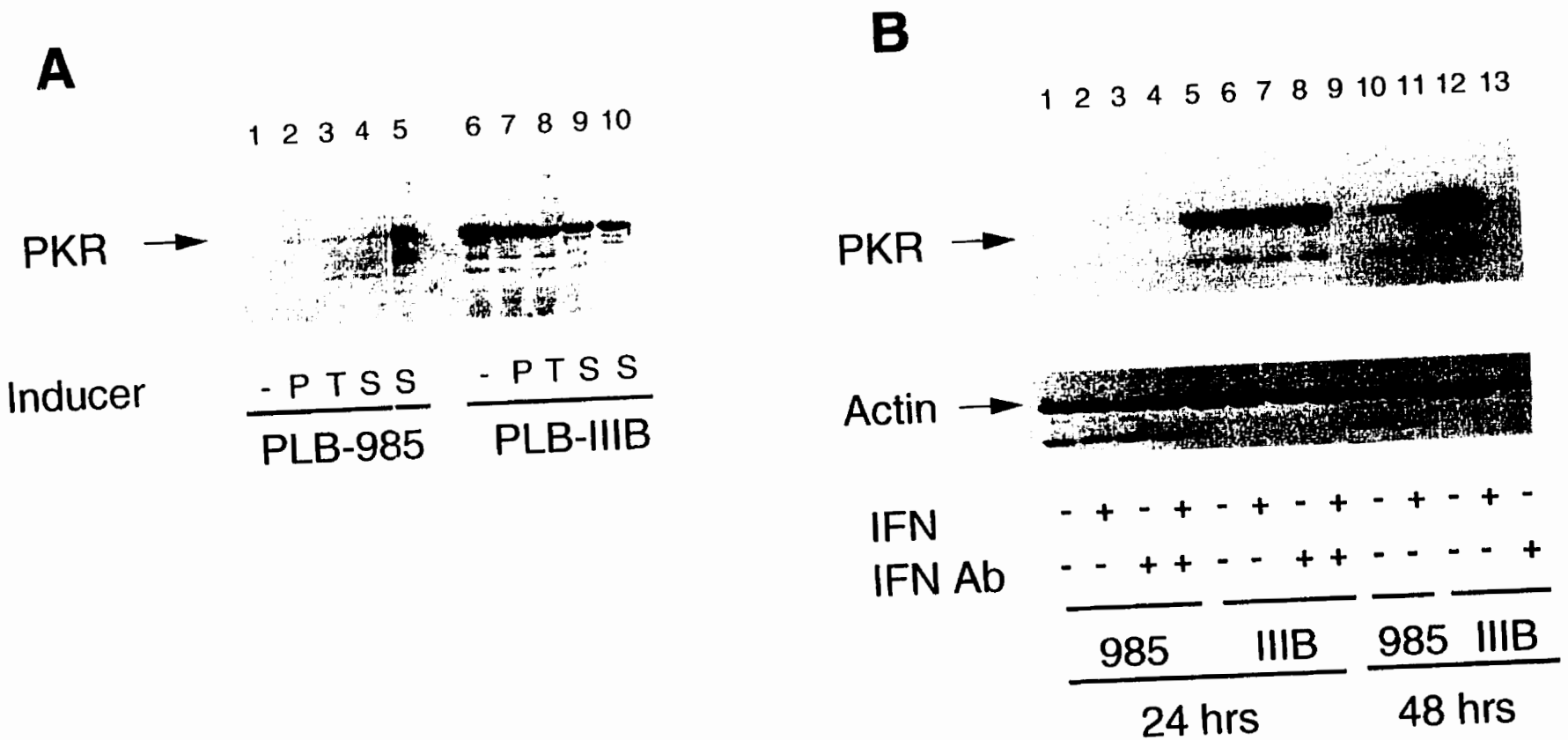

A mRNA
B Protein
C Activity
1 2 3 4 5 6
1 2 3 4
1 2 3 4
PKR
Induction
U P S U P S
U S U S
U S U S
PLB985 PLBIIIB PLB985 PLBIIIB PLB985 PLBIIIB

Figure 13. Modulation of PKR protein expression levels in PLB-985 and PLB-IIIB cells. (**A**) PLB-985 and PLB-IIIB cells were treated with PMA (P), TNFα (T) or were Sendai (S) virus infected for 0h (lanes 1 and 6), 6h (lanes 2-4 and 7-9) or 24h (lanes 5 and 10). PKR protein expression is identified by an arrow. (**B**) PLB-985 and PLB-IIIB cells were grown in the presence or absence of IFNα/β or anti-IFNα/β (IFN-Ab) for 24 or 48h. The immunoblot was reprobed for actin to confirm equal protein loading per lane. PKR and actin are identified by arrows.

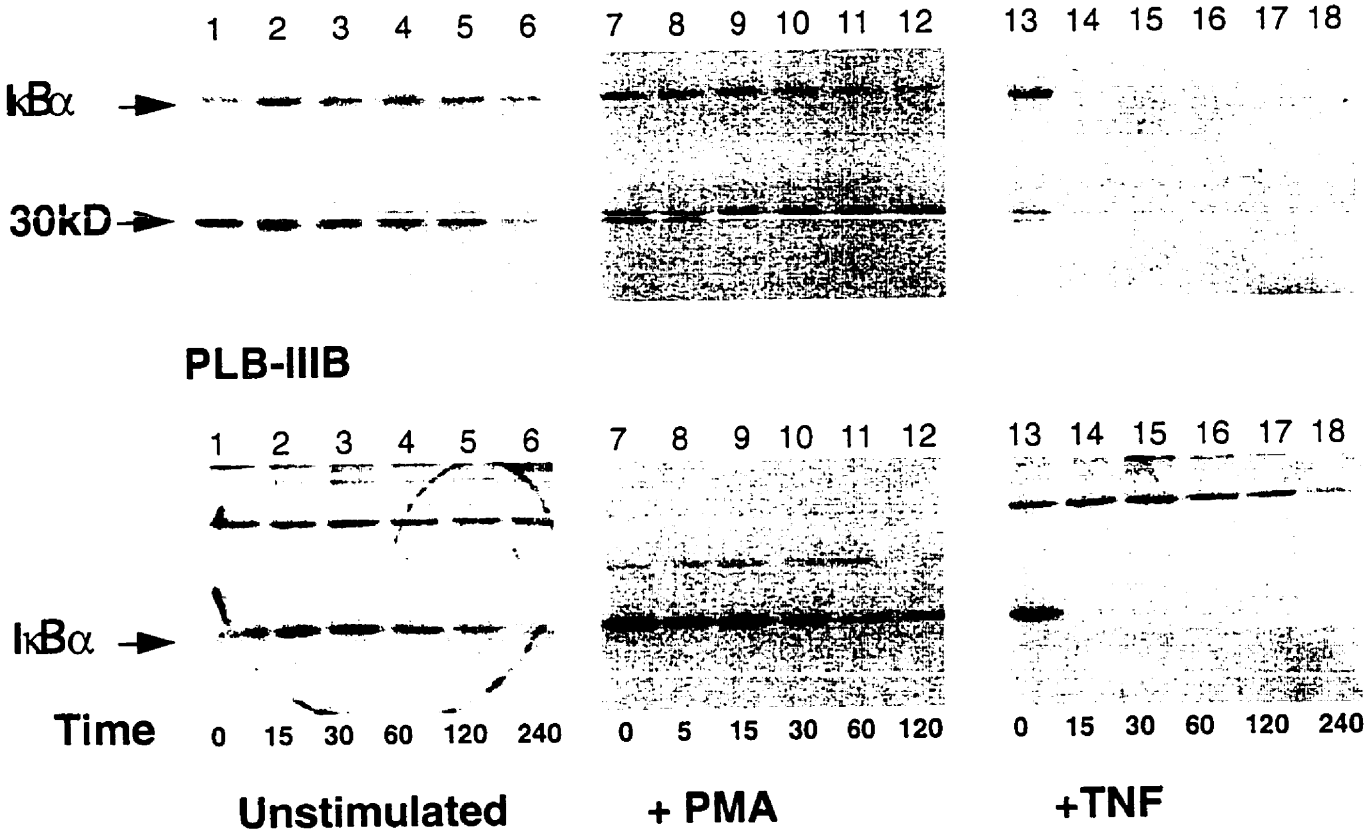

A
1 2 3 4 5 6 7 8 9 10
PKR
Inducer
- P T S S
- P T S S
PLB-985
PLB-IIIB
B
1 2 3 4 5 6 7 8 9 10 11 12 13
PKR
Actin
IFN
IFN Ab
985
IIIB
985
IIIB
24 hrs
48 hrs

expression levels in PLB-IIIB cells, 1000 NU/ml of anti-leukocyte IFNα/β (anti-IFN) antibody was added to the cultures. PLB-IIIB cells incubated in the presence of anti-IFN antibody for 48h, had dramatically reduced levels of PKR protein (Fig. 13B, lanes 11-13); this effect of anti-IFN antibody required greater than 24h to establish, since changes in PKR levels were not detected in PLB-IIIB cells after a 24h treatment with anti-IFN antibody (Fig. 13B, lanes 8 and 9). Together, these results demonstrate that chronic HIV-1 infection of PLB-985 cells resulted in low level IFNα/β production which in turn stimulated constitutive expression of PKR.

IκBα turnover in PLB-985 and PLB-IIIB cells. Next, the turnover of IκBα in PLB-985 and PLB-IIIB cells was examined by immunoblot analyses (Fig. 14). Although IκBα levels were slightly elevated in HIV-infected compared to uninfected cells, turnover rates were consistently higher in PLB-IIIB cells (compare PLB-985 and PLB-IIIB panels). These data were all normalized to the β-actin protein levels to ensure that overall protein degradation rates were not affected in the different extract prepearations. In HIV-infected cells, immunoblot analysis of IκBα levels demonstrated that the total pool of IκBα turned over with a half life of about 90 min in cycloheximide treated cells, whereas in uninfected cells the T1/2 of IκBα was about 160 min (compare Fig. 14A lanes 1-6 for PLB-985 and PLB-IIIB; plotted in Fig. 14B). PMA plus cycloheximide treatment reduced the half life of IκBα in PLB-985 and PLB-IIIB cells to 100 min and 50 min respectively (Fig 14A lanes 7-12; Fig. 14B). Treatment of both PLB-985 and PLB-IIIB with TNFα in the presence of cycloheximide resulted in rapid inducer-mediated degradation of IκBα with a T1/2 of <10 min (Fig 14, lanes 13-18; Fig. 15B). Interestingly, a 30-kDa AR20 immunoreactive band exhibiting similar degradation kinetics to IκBα, was present in PLB-985 cells but absent from PLB-IIIB cells. The 30-kDa form appears to be a degradation product of IκBα, although we cannot rule out the possiblity of a differentially spliced product or a distinct immunoreactive species. Similar turnover kinetics for IκBα in U937 cells and HIV-infected

Figure 14. IκBα turnover in PLB-985 and PLB-IIIB cells. (**A**) PLB-985 and PLB-IIIB cells were cultured in the presence of cycloheximide and were either untreated (U), PMA treated (P) or TNFα treated (T) for the times indicated. Protein extracts were resolved by SDS-PAGE, transferred to nitrocellulose and visualized using affinity purified IκBα antibody (AR20). The band corresponding to IκBα is identified by an arrow. (**B**) Autoradiograms were scanned by laser densitometry and the intensity of each band is presented in terms of % IκBα remaining in PLB-985 (□), PLB-985 plus PMA (■), PLB-985 plus TNFα (●), PLB-IIIB (○) PLB-IIIB plus PMA (▲) and PLB-IIIB plus TNFα (▲) cells compared to the total at time 0.

A PLB-985


1 2 3 4 5 6
7 8 9 10 11 12
13 14 15 16 17 18
IκBα
30kD
PLB-IIIB
1 2 3 4 5 6
7 8 9 10 11 12
13 14 15 16 17 18
IκBα
Time
0 15 30 60 120 240
0 5 15 30 60 120
0 15 30 60 120 240
Unstimulated
+ PMA
+TNF

B

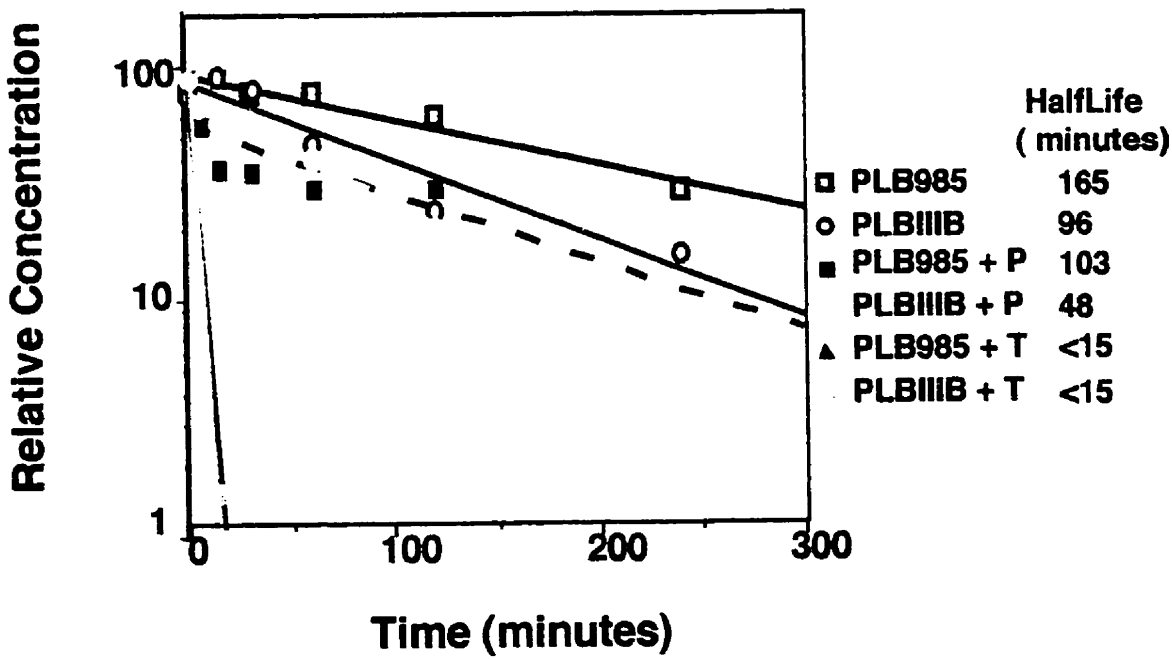
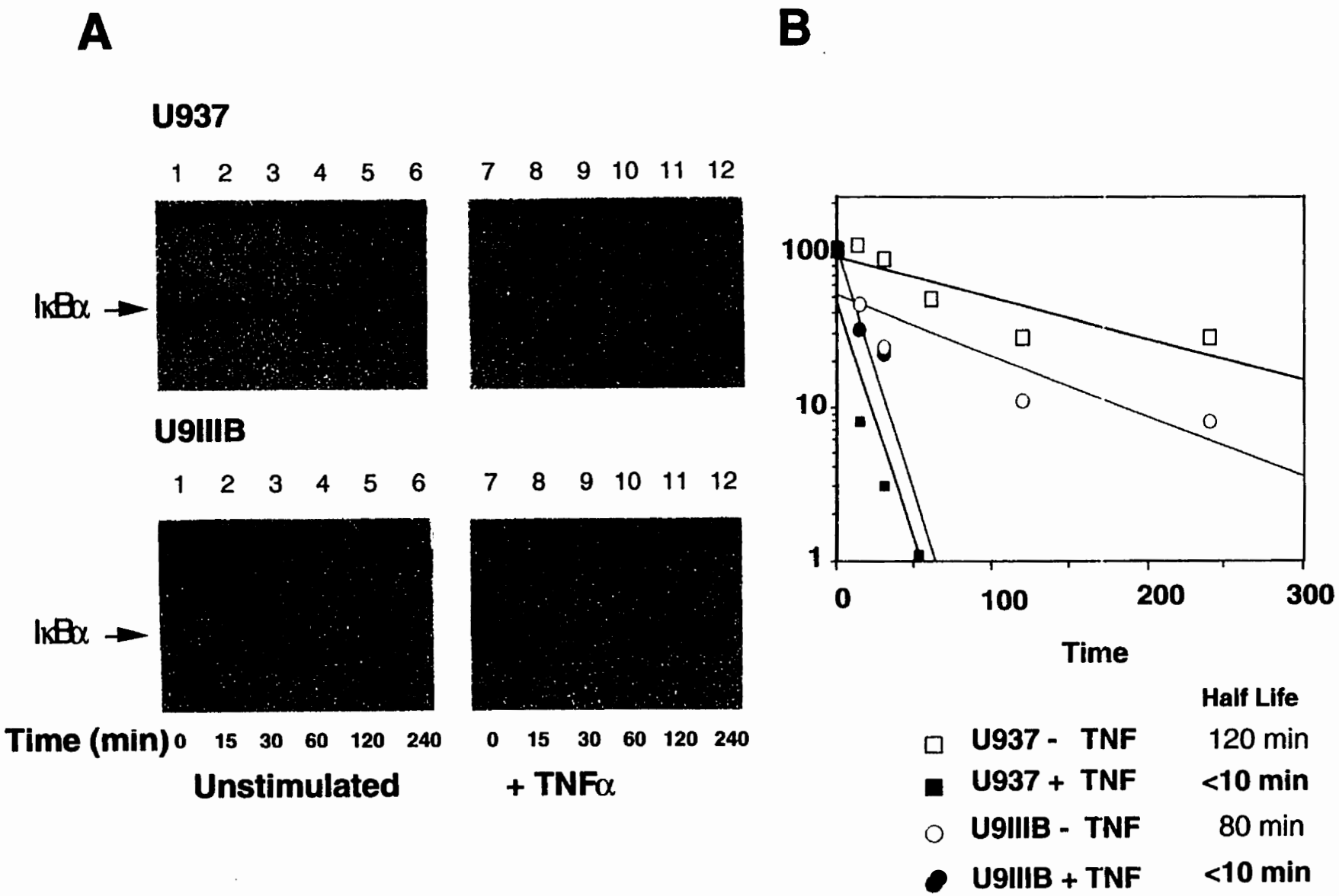

Relative Concentration
100
10
1
0
100
200
300
Time (minutes)
HalfLife
(minutes)
PLB985 165
PLBIIIB 96
PLB985 + P 103
PLBIIIB + P 48
PLB985 + T <15
PLBIIIB + T <15

Figure 15. IκBα turnover in U937 and U9-IIIB cells. (**A**) U937 and U9-IIIB cells were cultured in the presence of cycloheximide and were either untreated (U) or TNFα treated (T) for the times indicated. Protein extracts were resolved by SDS-PAGE, transferred to nitrocellulose and visualized using affinity purified IκBα antibody (AR20). The band corresponding to IκBα is identified by an arrow. (**B**) Autoradiograms were scanned by laser densitometry and the intensity of each band is presented in terms of % IκBα remaining in U937 (□), U937 plus TNFα (■), U9-IIIB (○) and U9-IIIB plus TNFα (●) cells compared to the total at time 0.

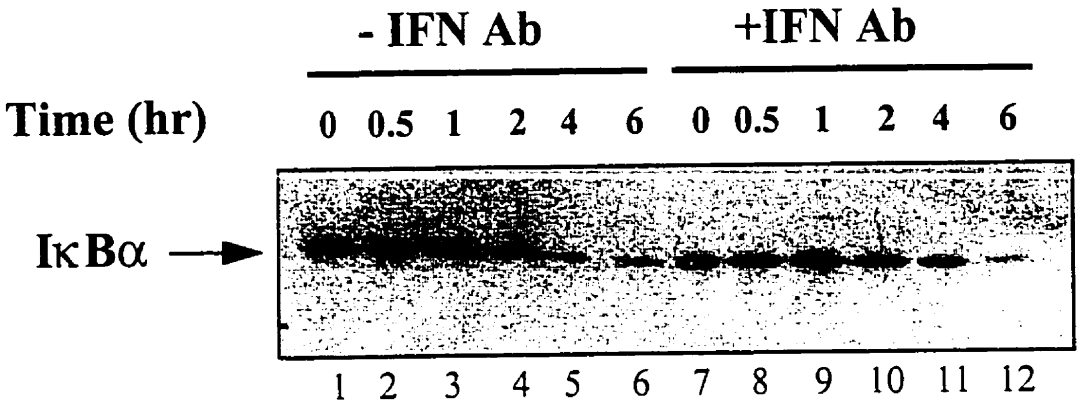

A
U937
1 2 3 4 5 6
7 8 9 10 11 12
IκBα
U9IIIB
1 2 3 4 5 6
7 8 9 10 11 12
IκBα
Time (min) 0 15 30 60 120 240
0 15 30 60 120 240
Unstimulated
+ TNFα
B
100
10
1
0
100
200
300
Time
Half Life
U937 - TNF 120 min
U937 + TNF <10 min
U9IIIB - TNF 80 min
U9IIIB + TNF <10 min

U9-IIIB cells were also observed (Fig. 15). In the presence of cycloheximide, IκBα had a half life of approximately 120 and 80 min in U937 cells and U9-IIIB cells respectively (compare Fig. 15A lanes 1-6 for U937 and U9-IIIB; Fig. 15B). As seen with PLB-985 and PLB-IIIB cells, TNFα reduced the turnover rate to < 10 min in both cell types (Fig. 15A lanes 7-12; Fig 15B).

Interestingly, the addition of anti-IFNα/β antibody to the culture medium for 48h altered the kinetics of IκBα turnover in PLB-IIIB cells (Fig. 16). Immunoblot analysis of IκBα levels (Fig. 16A) demonstrated that the total pool of IκBα degraded with a half-life of about 90 min in cycloheximide-treated PLB-IIIB cells whereas the $t_{1/2}$ of IκBα was about 160 min in anti-IFNα/β antibody treated cells (Fig. 16B), similar to the turnover rate in uninfected PLB-985 cells. These results indicate that constitutive PKR activity in HIV-infected cells may contribute to the overall turnover of the IκBα pool and that downregulation of PKR by interfering with the IFN-inductive signal may alter IκBα turnover in PLB-IIIB cells.

IκBα mRNA expression in PLB-985 and PLB-IIIB cells. We next sought to determine if the increased turnover rate of IκBα in HIV infected cells was accompanied by increased transcription of the IκB gene, as described previously (250). IκBα mRNA expression was examined by northern blot analyses and normalized to actin levels (Fig. 17). *Ikba* (MAD-3) mRNA levels were equivalent in unstimulated PLB-985 and PLB-IIIB cells when normalized to actin (Fig. 17A, lanes 1,2 and 6,7) and were inducible by PMA treatment or Sendai virus infection by approximately 25-fold in PLB-985 and 5-15 fold in PLB-IIIB (Fig. 17, lanes 3-5 and 8-10). In other experiments, IκBα mRNA levels were stimulated as early as 2h after treatment in PLB-IIIB cells and by 8h after induction IκBα mRNA levels were close to unstimulated levels (data not shown). In contrast, PLB-985 cells exhibited maximal IκBα mRNA induction at 6h. Thus, in response to increased NF-κB binding activity in HIV-infected cells, the IκBα/MAD3 gene was transcriptionally

Figure 16. IκBα turnover in PLB-IIIB cells treated with neutralizing anti-IFN antibody. (**A**) PLB-IIIB cells were either untreated (lanes 1-6) or treated with anti-IFN antibody (lanes 7-12) at 1 000 IU/ml for 48h. Cells were then cultured in the presence of cycloheximide for different times, protein extracts (20 μg) were resolved by SDS-PAGE, transferred to nitrocellulose, and visualized with affinity-purified IκBα antibody (AR20), as described in the legend to Fig. 4. (**B**) Autoradiograms were scanned by laser densitometry and the intensity of IκBα remaining at different times after the cycloheximide addition is presented as percentage of IκBα remaining in untreated PLB-IIIB cells (■) or PLB-IIIB cells treated with anti-IFN antibody (□).


- IFN Ab
+IFN Ab
Time (hr)
0 0.5 1 2 4 6 0 0.5 1 2 4 6
IκBα
1 2 3 4 5 6 7 8 9 10 11 12

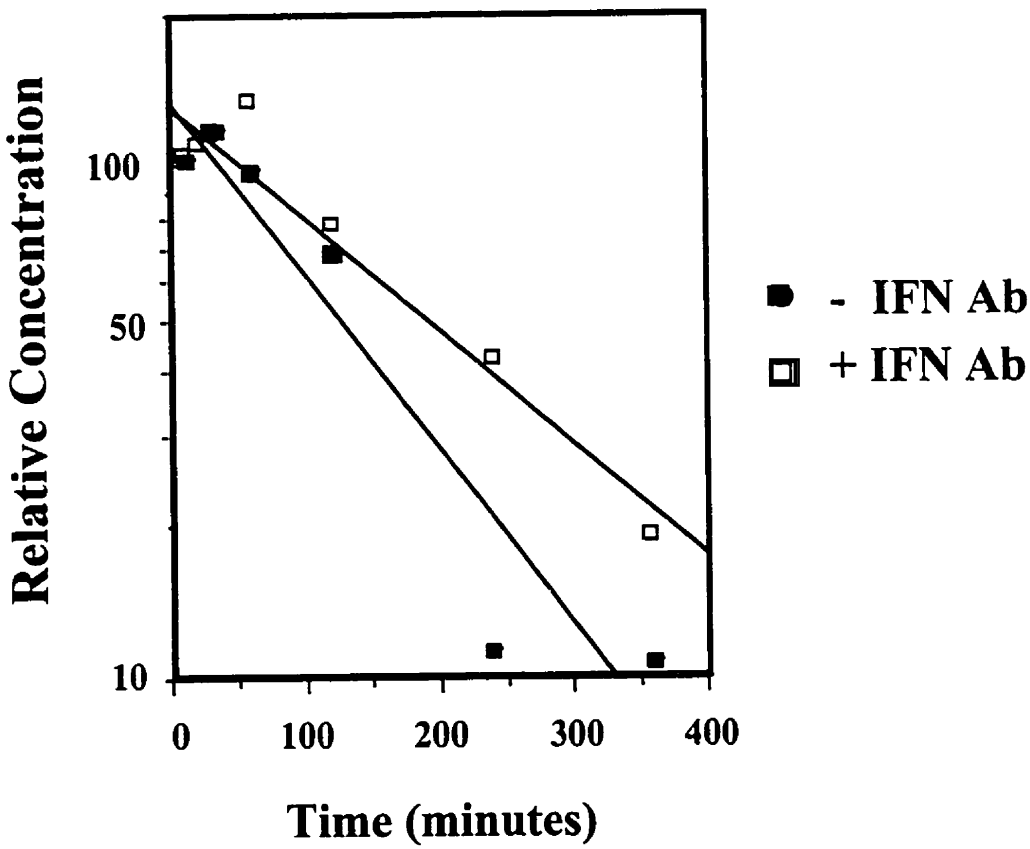
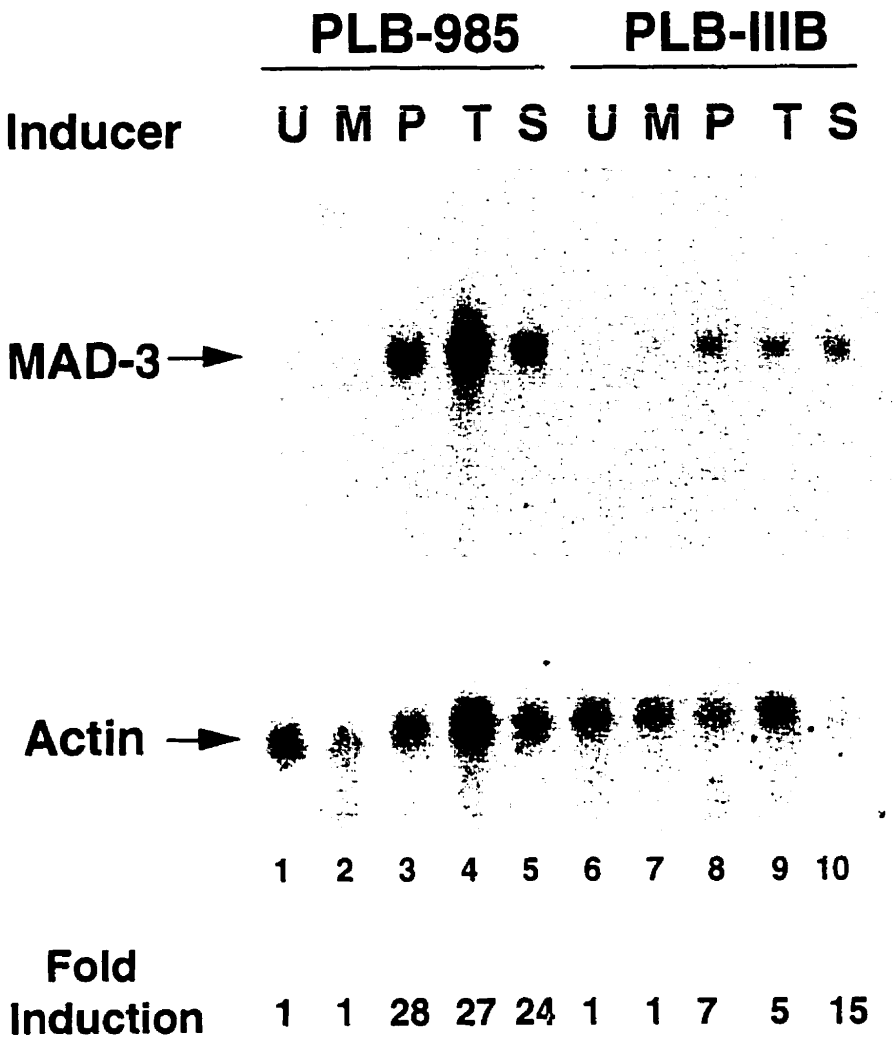

Relative Concentration
100
50
10
0
100
200
300
400
Time (minutes)
- IFN Ab
+ IFN Ab

Figure 17. Northern blot analysis of IκBα (MAD-3) mRNA levels in PLB-985 and PLB-IIIB cells. Total cellular RNA was isolated from PLB-985 (lanes 1 to 5) and PLB-IIIB (lanes 6 to 10) cells that were untreated (U), mock infected (M), PMA treated (P), TNFα (T) treated or Sendai virus infected (S) for 6h. RNA was separated on a 1% denaturing agarose gel containing ethidium bromide, and visualized under UV light. After RNA was transferred to a nylon membrane, the blot was probed with an IκBα cDNA probe. The blot was subsequently stripped and reprobed using an actin cDNA probe. The positions of the IκBα RNA mRNA MAD-3 (top panel) and the actin mRNA (lower panel) are identified by arrows. The fold induction after normalization to the actin signal is indicated below each lane.


PLB-985
PLB-IIIB
Inducer
U M P T S U M P T S
MAD-3
Actin
1 2 3 4 5 6 7 8 9 10
Fold Induction
1 1 28 27 24 1 1 7 5 15

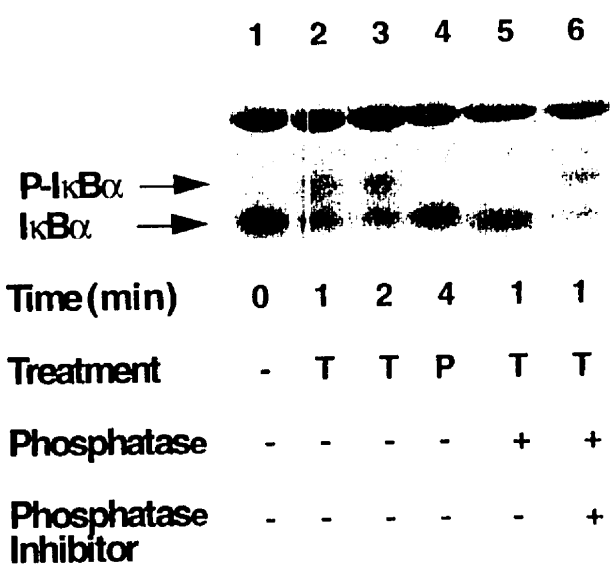
induced in both cells types. However, in PLB-IIIB cells, induction of IκBα transcription appears to be decreased in response to various stimuli (Fig. 17). This modulation in the level of IκBα gene induction may reflect differences in the relative abundance and transactivation potential of the constitutive NF-κB binding activity in HIV-infected cells (see below).

Phosphorylation of IκBα. To examine the comparative degree of phosphorylation of IκBα in PLB-985 and PLB-IIIB, IκBα was analysed at different times following treatment with TNFα or PMA (Fig. 18). Phosphorylated IκBα was detected by immunoblot analysis in both PLB-985 (data not shown) and PLB-IIIB cells treated with TNFα as early as one or two min after stimulation (Fig. 18A, lanes 2 and 3). The slower migrating band, present only in TNFα stimulated cells, was lost when extracts were incubated with potato acid phosphatase (Fig. 18A, lane 5) and was restored when incubated with phosphatase and phosphatase inhibitors (Fig. 18A, lane 6), thus confirming that the upper band represented a phosphorylated form of IκBα. PMA treatment for four min did not result in the appearance of phosphorylated IκBα (compare Fig. 18A, lanes 4 and 2), indicating that TNFα was a more potent inducer of IκBα phosphorylation than PMA. This observation is also consistent with the more modest effect of PMA on IκBα turnover (see Fig.14).

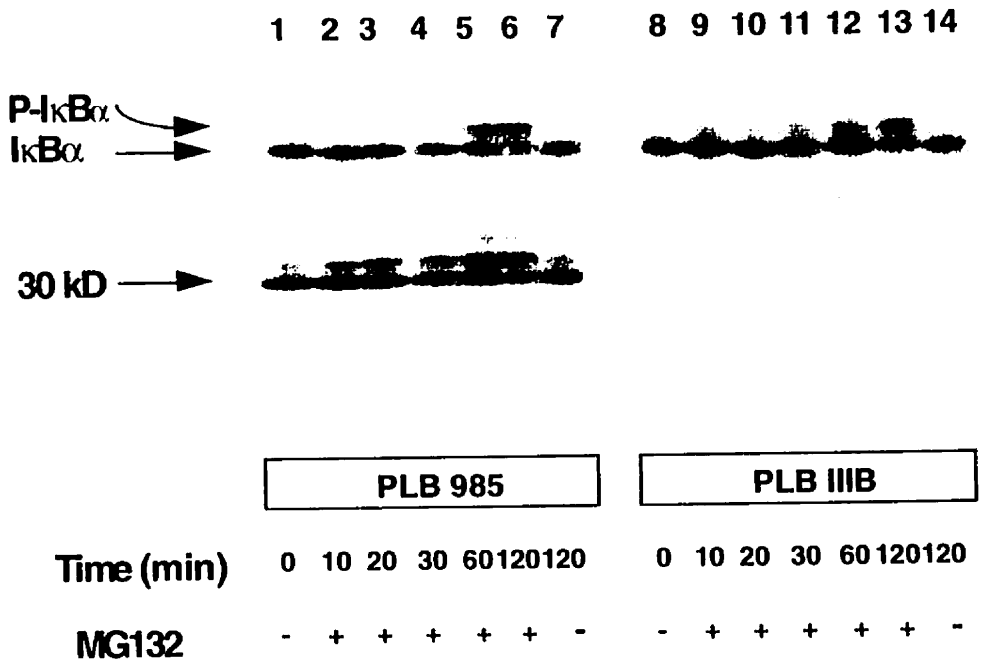
The constitutive turnover rate of IκBα protein was increased in PLB-IIIB compared to PLB-985 cells (see Fig. 14). To determine if this increase was related to an enhanced constitutive phosphorylation in HIV-infected cells, the rate of accumulation of phosphorylated IκBα was analyzed in the presence of the proteasome inhibitor MG132 (Fig. 18B). Using this approach, IκBα phosphorylation in PLB-985 (Fig. 18B, lanes 2-6) and PLB-IIIB (Fig. 18B, lanes 8-13) appeared to be similar. Also, extracts from PLB-985 or PLB-IIIB cells (Fig. 18B, lanes 7 and 14) incubated with DMSO for two hours resembled control extracts, confirming that accumulation of phosphorylated IκBα was not

Figure 18. Analysis of IκBα phosphorylation. (A) PLB-IIIB cells were stimulated with TNFα (T) for 1 or 2 min (lanes 2 and 3) or PMA (P) for 4 min (lane 4). Cell extracts were prepared and TNFα treated samples (1 min) were then subjected to potato acid phosphatase treatment (lane 5) or potato acid phosphatase treatment plus inhibitors (lane 6) for 30 minutes. Equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose and visualized using affinity purified IκBα antibody (AR20). TNFα induced phosphorylation was compared to control levels (lane 1). **(B)** The accumulation of phosphorylated IκBα (P-IκBα) was analyzed in PLB-985 cells (lanes 1-7) and PLB-IIIB cells (lanes 8-14) incubated with 25 μM MG132 (lanes 2-6 and lanes 9-13 respectively) or DMSO (lane 7 and lane 14) for the indicated times. IκBα, P-IκBα and the 30 kDa band, detected using a monoclonal IκBα antibody, are indicated by arrows.

A


1 2 3 4 5 6
P-IκBα
IκBα
Time (min) 0 1 2 4 1 1
Treatment - T T P T T
Phosphatase - - - - + +
Phosphatase Inhibitor - - - - - +

B


1 2 3 4 5 6 7 8 9 10 11 12 13 14
P-IκBα
IκBα
30 kD
PLB 985
PLB IIIB
Time (min) 0 10 20 30 60 120 120 0 10 20 30 60 120 120
MG132 - + + + + + - - + + + + + -

due to DMSO treatment but rather to stabilization of the phosphorylated product by MG132. Interestingly, the 30-kDa IκBα–like band specific to PLB-985, was highly phosphorylated at time zero (Fig. 18B, lane 2 lower band); at later times two distinct slowly migrating forms of 30-kDa IκBα were detectable (Fig. 18B, lanes 5 and 6).

NF-κB/Rel protein expression in PLB-985 and PLB-IIIB cells. The altered kinetics of IκBα turnover in PLB-IIIB cells suggested that expression of NF-κB subunits may be similarly altered in HIV-infected cells. In previous studies composition of NF-κB·DNA complexes was analyzed but the relative abundance of different NF-κB subunits was not evaluated. To examine the protein levels of various NF-κB·DNA-binding proteins in HIV-infected and control PLB-985 cells, immunoblot analyses with NF-κB subunit specific antibodies were performed and normalized by analysis of β-actin levels (Fig. 19 and 20). Protein extracts were prepared from PLB-985 and PLB-IIIB cells induced for 8, 16 or 22h with PMA or TNFα. A representative summary of the immunoblot analyses of NF-κB1 and NF-κB2 proteins is shown in Figure 19 and illustrates that the levels NF-κB2 p100 and p52 (Fig. 19A and 19B) were higher in unstimulated PLB-IIIB cells compared to unstimulated PLB-985 cells. HIV-infected cells had approximately 3-4 fold more p100 and 2 fold more p52 than uninfected PLB-985 cells. In contrast, NF-κB1 p105 and p50 levels (Fig. 19C and 19D) were not substantially increased. Stimulation with PMA or TNFα (Fig 19 and data not shown) had little effect on NF-κB1 p105 and p50 levels but resulted in enhanced levels of NF-κB2 p100 (Fig. 19A). RelA levels were about 2 fold higher in PLB-IIIB cells compared to PLB-985 cells and levels were not dramatically altered by PMA or TNFα treatment (Fig. 20C and 20D). In contrast, the levels of c-Rel were at least 8 fold higher in infected cells compared to non-infected cells (Fig. 20A and 20B). Following induction with PMA or TNFα, c-Rel levels were up to 50 fold higher than levels detected in unstimulated PLB-985 and 2-3 fold higher than c-Rel protein in unstimulated PLB-IIIB cells (Fig. 20A and 20B). Interestingly, stimulation with PMA or TNFα for 22h (Fig. 20B

and data not shown) led to sustained expression of c-Rel in PLB-IIIB cells, whereas in PLB-985 cells, c-Rel levels returned to control levels. Other studies also demonstrated that NF-κB subunit expression was similarly increased in U9-IIIB compared to U937 cells although unlike PLB-IIIB cells, U9-IIIB cells exhibited increased levels of p105 and p50 (data not shown). These results suggest that intracellular pools of NF-κB subunits are increased in HIV-1 chronically infected myeloid cells.

Figure 19. Analysis of NF-κB1 and NF-κB2 protein expression in PLB-985 and PLB-IIIB cells. Cell extracts isolated by detergent lysis from PLB-985 and PLB-IIIB cells left untreated (☐) or stimulated with PMA for 8 or 22h (▩) were resolved by SDS-PAGE, transferred to a nitrocellulose membrane and blotted with NF-κB-specific antisera: (**A**) p100, (**B**) p52, (**C**) p105, and (**D**) p50. The specific signals were detected using the ECL chemiluminescence detection system. Autoradiograms were scanned by laser densitometry and the intensity of the each band is plotted in terms of relative protein levels. Due to affinity differences between NF-κB antibodies, the relative amounts of the different proteins cannot be compared.

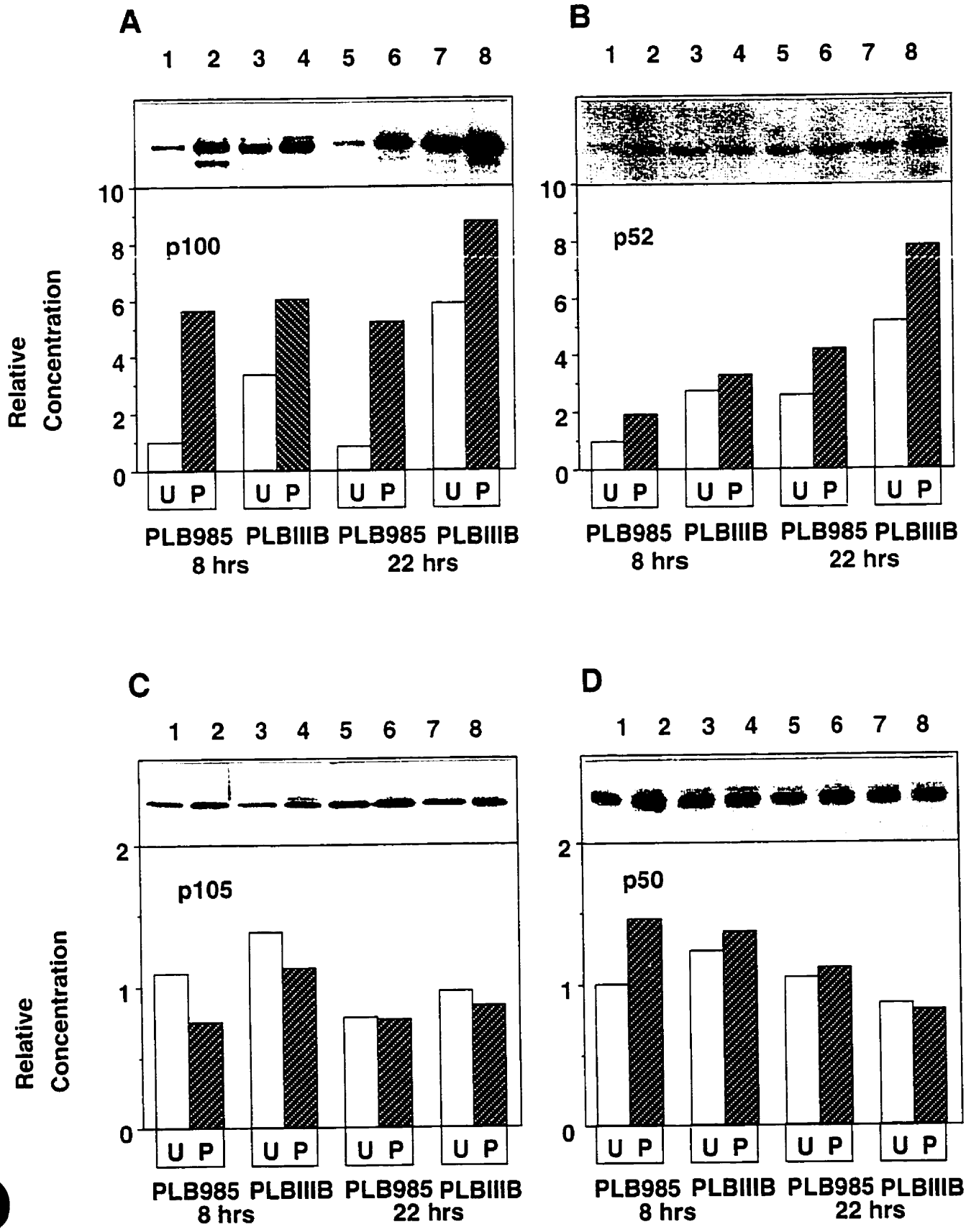
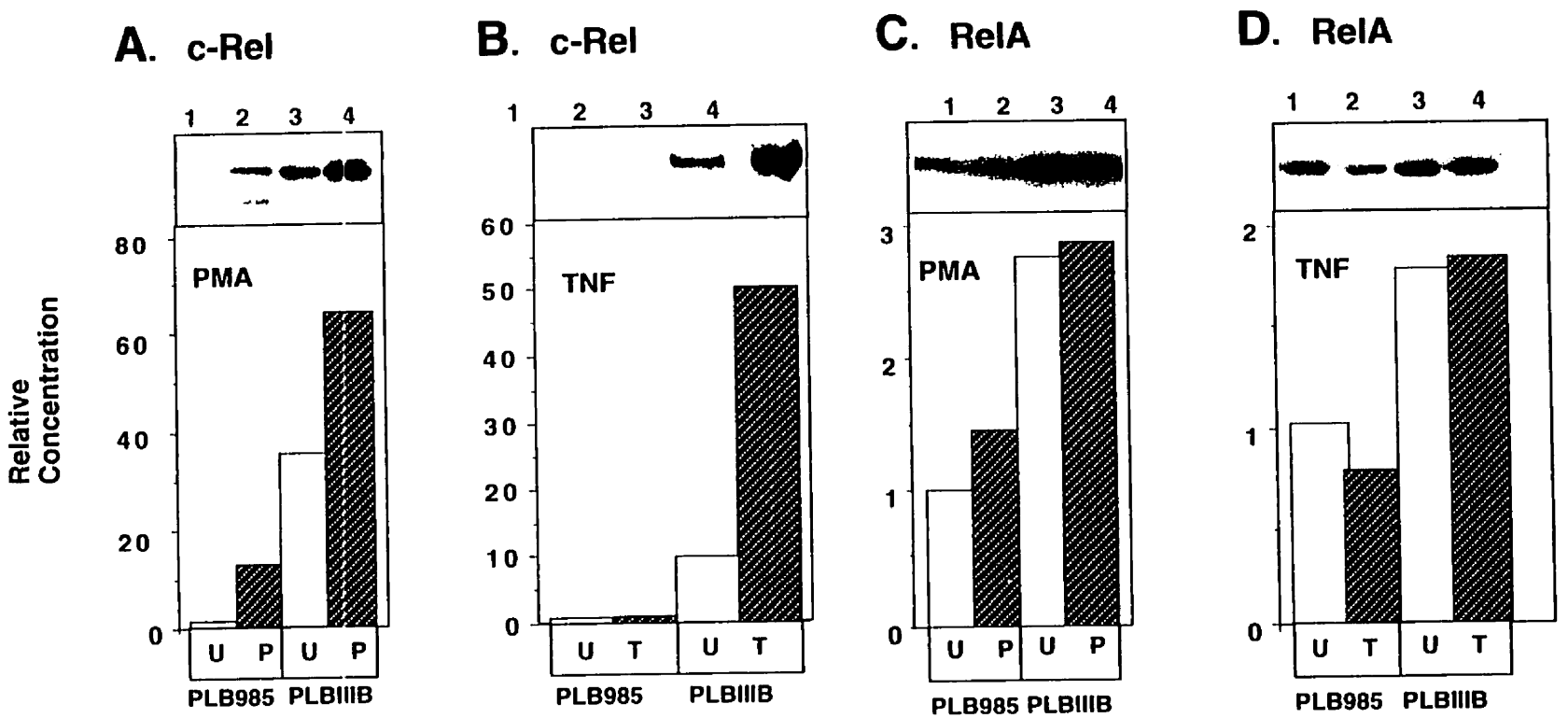

A
1 2 3 4 5 6 7 8
p100
Relative Concentration
10
8
6
4
2
0
U P U P U P U P
PLB985 PLBIIIB PLB985 PLBIIIB
8 hrs 22 hrs
B
1 2 3 4 5 6 7 8
p52
10
8
6
4
2
0
U P U P U P U P
PLB985 PLBIIIB PLB985 PLBIIIB
8 hrs 22 hrs
C
1 2 3 4 5 6 7 8
p105
Relative Concentration
2
1
0
U P U P U P U P
PLB985 PLBIIIB PLB985 PLBIIIB
8 hrs 22 hrs
D
1 2 3 4 5 6 7 8
p50
2
1
0
U P U P U P U P
PLB985 PLBIIIB PLB985 PLBIIIB
8 hrs 22 hrs

Figure 20. Analysis of c-Rel and RelA expression in PLB-985 and PLB-IIIB cells. Cell extracts isolated by detergent lysis from PLB-985 and PLB-IIIB cells left untreated (□), treated with PMA for 16h or TNFα for 22h (■) were resolved by SDS-PAGE, transferred to nitrocellulose and blotted with NF-κB-specific antisera: (A) c-Rel -/+ PMA, (B) c-Rel -/+ TNFα, (C) RelA -/+ PMA, and (D) RelA -/+ TNF α.


A. c-Rel
B. c-Rel
C. RelA
D. RelA
Relative Concentration
1 2 3 4
PMA
TNF
80
60
40
20
0
50
30
10
3
2
1
U P U P
U T U T
PLB985 PLBIIIB

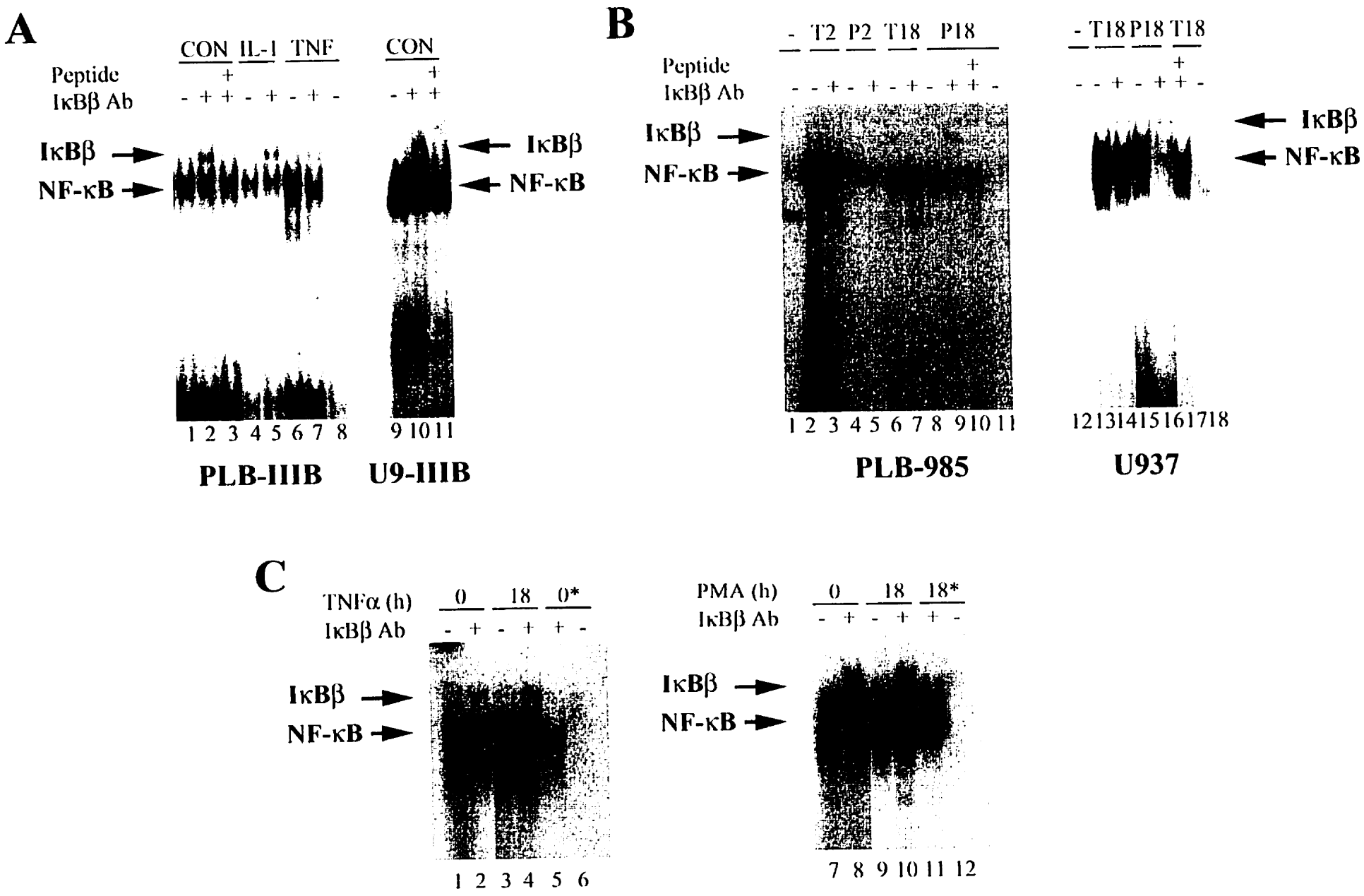
CHAPTER IV

NUCLEAR IκBβ MAINTAINS PERSISTENT NF-κB ACTIVATION IN HIV-1 INFECTED MYELOID CELLS

IκBβ is part of the NF-κB·DNA binding complex in HIV-infected cells. Previous studies demonstrated that myeloid cell lines PLB-985 and U937 acquire constitutive NF-κB·DNA binding activity upon HIV-1 infection (25,112,370,372). Analysis of this complex revealed that the DNA binding activity was composed predominantly of RelA and p50 heterodimers with a minor contribution of c-Rel and p50 heterodimers (113,370,372). To investigate the possibility that IκBβ may be involved in maintaining this persistent activation, nuclear extracts prepared from HIV-infected PLB-IIIB and U9-IIIB cells were analysed for DNA binding levels by EMSA. Analysis of NF-κB·DNA binding activity using an IκBβ specific antibody that recognized the 43-kDa isoform of IκBβ demonstrated that IκBβ protein was a part of the DNA binding complex (Fig 21A, lanes 2 and 10) and could be detected in cells stimulated with TNFα or IL-1β for 6h (Fig 21A, lanes 5 and 7). Pre-incubation with the cognate peptide recognized by the IκBβ antibody demonstrated the specificity of antibody recognition (Fig 21A, lanes 3 and 11), while incubation with excess unlabeled NF-κB probe competed the NF-κB specific complexes (Fig 21A, lane 8). Similar experiments using IκBα did not produce a shifted complex (data not shown), suggesting that IκBβ was uniquely present in HIV-infected cells. Uninfected PLB-985 and U937 cells stimulated with TNFα or PMA for 18h (Fig 21B, lanes 9, 14 and 16) - but not cells treated for shorter times (Fig 21B, lanes 3 and 5) - likewise exhibited an NF-κB·DNA binding complex that could be supershifted with IκBβ antibody. Induction of PLB-IIIB cells with TNFα or PMA for 0, 2, 4, 8, 12 or 18h revealed that IκBβ remained part of the DNA binding complex over the course of induction (Fig 21C and data not shown).

The presence of IκBβ in the nuclear compartment of HIV-infected myeloid cells was confirmed by biochemical fractionation; cytoplasmic and nuclear extracts from PLB-IIIB and PLB-985 cells stimulated with TNFα or PMA were separated by SDS-PAGE and immunoblotted for IκBβ. Whereas IκBβ was present in the cytoplasm and nucleus of

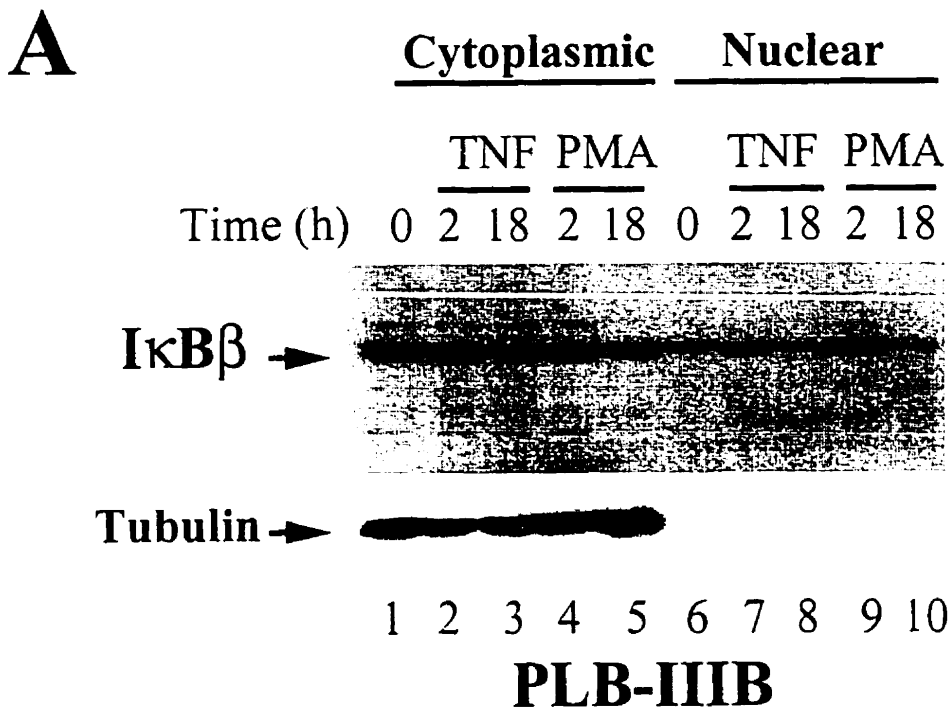
Figure 21. EMSA analysis of HIV-1 infected myeloid cells for IκBβ containing NF-κB·DNA binding complexes. (**A**) Nuclear extracts from PLB-IIIB cells were subjected to supershift analysis using an IκBβ specific antibody. Extracts were either unstimulated (lane 1-3) or stimulated for 6h with IL-1β (lane 4-5) or TNFα (lane 6-7). IκBβ supershifted bands are identified by the upper arrow. Antibody specificity was demonstrated using cognate peptide (lane 3) and probe specificity by competition with excess unlabeled probe (lane 8). Unstimulated U9-IIIB nuclear extract (lanes 9-11) was incubated with IκBβ antibody (lane 10) or antibody and peptide (lane 11) and analyzed as above. (**B**) Nuclear extracts from PLB-985 cells or U937 cells were unstimulated (lane 1 and 12), stimulated with TNFα for 2h (T 2h, lanes 2-3) or 18h (T 18h, lanes 6,7,13,14, 17 and 18) or stimulated with PMA for 2h (P 2h, lanes 4-5) or 18h (P 18h, lane 8, 9, 15 and 16). Extracts were incubated alone (lanes 1, 2 , 4, 6, 8, 11, 12, 13, 15 and 18), with IκBβ antibody (lanes 3, 5, 7, 9, 14 and 16) or with IκBβ antibody and peptide (lane 10 and 17). PMA stimulated extract (lane 11) or TNFα induced extract (lane 18) were competed with cold probe. (**C**) PLB-IIIB cells were stimulated with TNFα (lanes 1-6) or PMA (lanes 7-12) for 0 or 18h. Extracts were incubated alone (lanes 1, 3, 7 and 9), or with IκBβ antibody (lanes 2, 4, 8 and 10) or IκBβ antibody and peptide (*, lanes 5 and 11). IκBβ containing complexes are identified with an arrow. Specificity was confirmed by incubating with excess unlabeled probe (* lanes 6 and 12).


A
CON IL-1 TNF
Peptide +
IκBβ Ab - + + - + - + -
IκBβ
NF-κB
1 2 3 4 5 6 7 8
PLB-IIIB
CON
+
- + +
IκBβ
NF-κB
9 10 11
U9-IIIB
B
- T2 P2 T18 P18
Peptide +
IκBβ Ab - - + - + - + - + + -
IκBβ
NF-κB
1 2 3 4 5 6 7 8 9 10 11
PLB-985
- T18 P18 T18
+
- - + - + + -
IκBβ
NF-κB
12 13 14 15 16 17 18
U937
C
TNFα (h) 0 18 0*
IκBβ Ab - + - + + -
IκBβ
NF-κB
1 2 3 4 5 6
PMA (h) 0 18 18*
IκBβ Ab - + - + + -
IκBβ
NF-κB
7 8 9 10 11 12

HIV-infected cells (Fig 22A, lanes 1 and 6), IκBβ was predominantly cytoplasmic in non-infected PLB-985 cells (Fig 22B, lane 1 and 6). IκBβ was present in the nucleus of HIV-infected cells after stimulation with TNFα or PMA (Fig 22A, lanes 7-10) and low levels were also detected in stimulated PLB-985 cells (Fig 22B, lanes 7-10). Nuclear extracts were shown to be free of cytoplasmic contamination by reprobing with α-tubulin antibody (Fig 22A and 22B, lower panels). Similar results were obtained with U937 and U9-IIIB cells (data not shown).

IκBβ protects NF-κB·DNA complexes from IκBα mediated dissociation. Since previous *in vitro* studies have demonstrated that NF-κB·DNA complexes are sensitive to dissociation by IκBα (19,480), the possibility that IκBβ protects NF–κB·DNA complexes from IκBα dissociation was evaluated. The NF–κB·DNA binding complex from HIV-infected U9-IIIB cells was resistant to GST-IκBα mediated dissociation (Fig 23A, lanes 1-3); furthermore GST-IκBα did not reduce NF-κB binding to levels less than those observed in unstimulated HIV-infected cells (Fig 23A, lanes 4-15). Similar results were obtained with PLB-IIIB cells (Fig 23B) suggesting this phenomenon was a property of HIV-infected myeloid cells. In contrast, NF–κB·DNA complexes from TNFα or PMA stimulated PLB-985 and U937 cells were completely dissociated by GST-IκBα (Fig 23C and 23D, lanes 2, 3, 5 and 6).

Figure 22. IκBβ is found in the nucleus of HIV-1 infected cells. PLB-IIIB (**A**) or PLB-985 cells (**B**) were treated with TNFα (lanes 2, 3 and 7,8) or PMA (lanes 4, 5 and 9, 10) for 0, 2 or 18h. Cytoplasmic extract (40 μg) (lanes 1-5) or nuclear extract (40 μg) (lanes 6-10) were separated by SDS-PAGE and immunoblotted for IκBβ (upper panels). The blots were stripped and reprobed for α-tubulin (lower panels).


A
Cytoplasmic
Nuclear
TNF PMA TNF PMA
Time (h) 0 2 18 2 18 0 2 18 2 18
IκBβ
Tubulin
1 2 3 4 5 6 7 8 9 10
PLB-IIIB

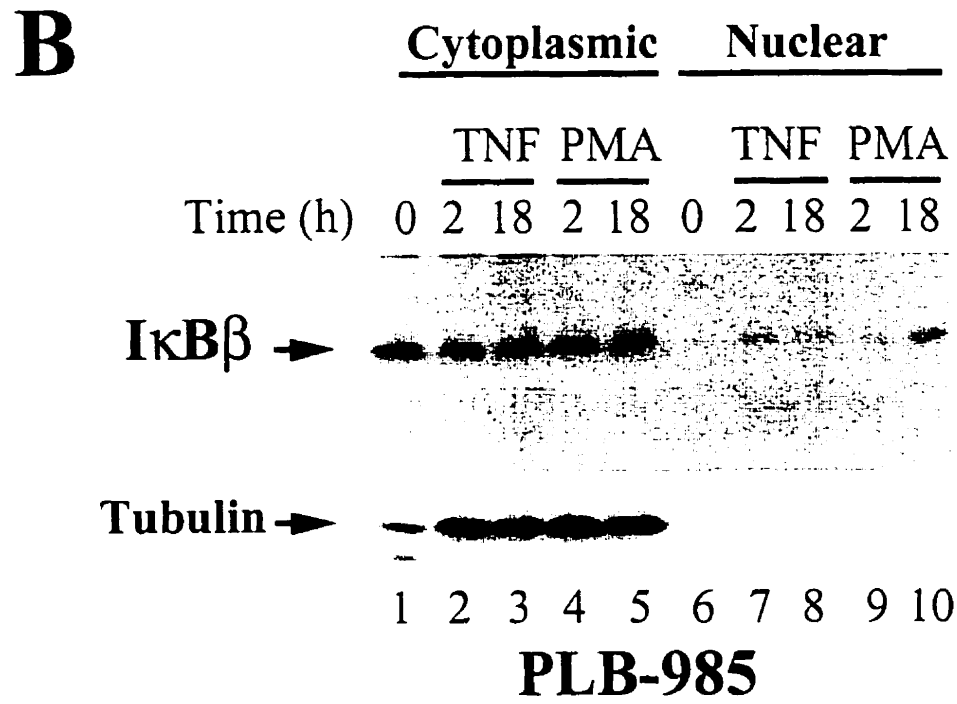
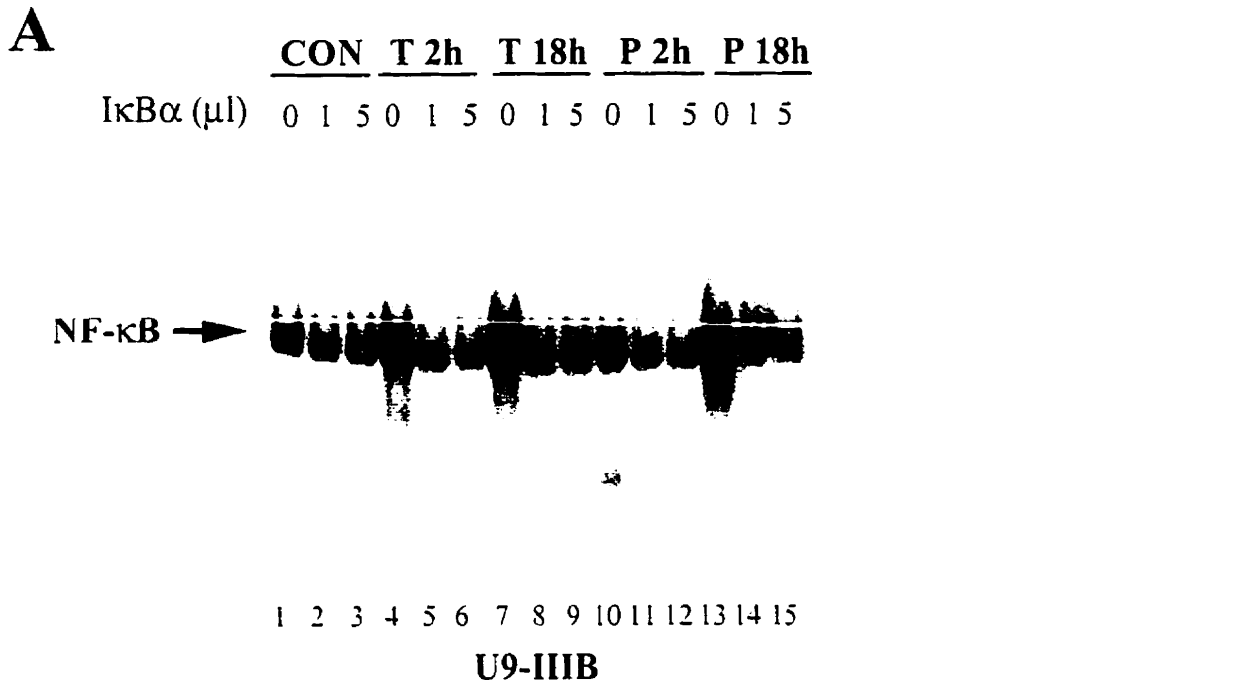

B
Cytoplasmic
Nuclear
TNF PMA TNF PMA
Time (h) 0 2 18 2 18 0 2 18 2 18
IκBβ
Tubulin
1 2 3 4 5 6 7 8 9 10
PLB-985

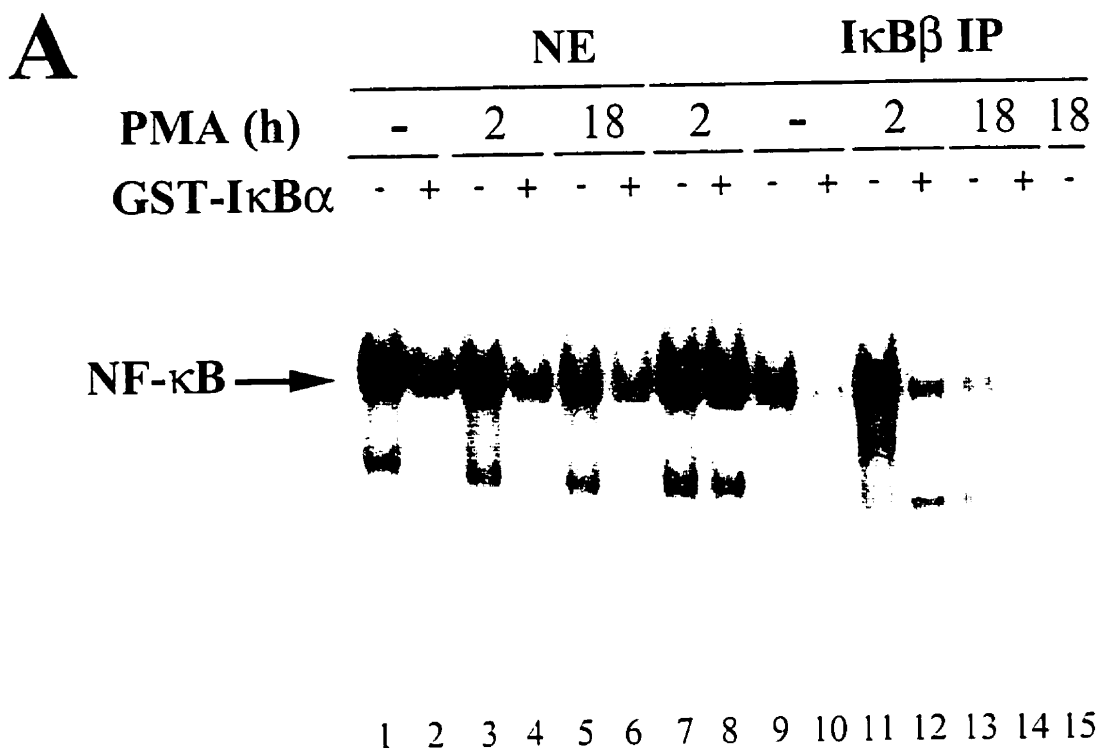
Figure 23. Recombinant IκBα does not dissociate preformed NF-κB·DNA complexes in HIV-1 infected cells. U9-IIIB (**A**) and PLB-IIIB (**B**) cells were untreated (CON, lanes 1-3), treated with TNFα for 2h (T 2h, lanes 4-6) or 18h (T 18h, lanes 7-9) or PMA for 2h (P 2h, lanes 10-12) or 18h (P 18h, lanes 13-15). Nuclear extracts were incubated with labeled NF-κB probe (PRDII element of IFN-β gene) followed by incubation with increasing amounts of GST-IκBα. PLB-985 (**C**) and U937 (**D**) cells were stimulated with TNFα for 2h (T 2h, lanes 1-3) or 18h (T 18h, lanes 4-6) and treated as described above.

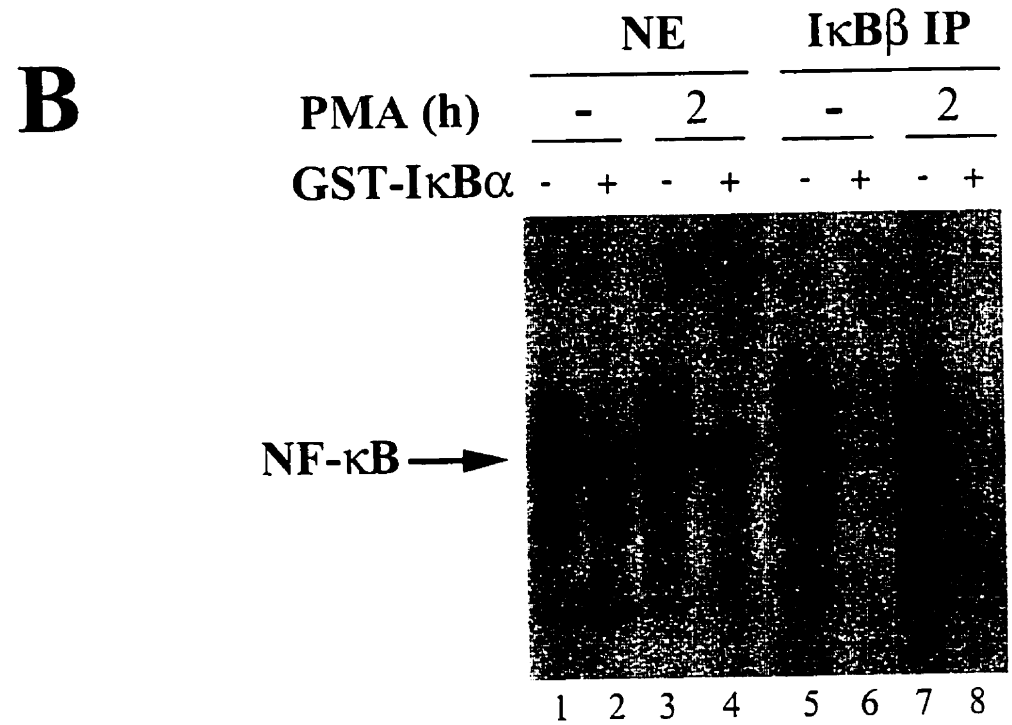

A
CON T 2h T 18h P 2h P 18h
IκBα (μl) 0 1 5 0 1 5 0 1 5 0 1 5 0 1 5
NF-κB
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
U9-IIIB
B
CON T 2h
IκBα (μl) 0 1 5 0 1 5
NF-κB
1 2 3 4 5 6
PLB-IIIB
C
T 2h T 18h
IκBα (μl) 0 1 5 0 1 5
NF-κB
1 2 3 4 5 6
PLB-985
D
T 2h T 18h
IκBα (μl) 0 1 5 0 1 5
NF-κB
1 2 3 4 5 6
U937

Nuclear extracts were next immunodepleted of IκBβ using an IκBβ specific antibody and analysed for NF-κB·DNA binding. Incubation of PMA stimulated U9-IIIB nuclear extracts with GST-IκBα reduced the amount of NF-κB·DNA binding complexes, but did not completely dissociate NF-κB binding activity (Fig. 24A, lanes 1 - 6). In contrast, extracts immunodepleted for IκBβ were sensitive to GST-IκBα mediated dissociation (Fig 24A, lanes 9-14). In this case, levels of NF-κB binding were reduced to levels observed in uninfected U937 cells (Fig 23D), indicating that IκBβ played a role in maintaining NF-κB·DNA binding activity in infected cells. Furthermore, the use of control serum for the immunodepletion step did not increase the sensitivity of the protein-DNA complex to IκBα mediated dissociation (Fig 24A, lanes 7 and 8), indicating that the effect was specific. Similar results were obtained with untreated and PMA induced PLB-IIIB cells (Fig 24B). Like U9-IIIB cells, IκBβ depleted PLB-IIIB extracts were sensitive to GST-IκBα mediated dissociation of NF-κB·DNA binding activity (Fig 24B, compare lanes 2 and 4, 6 and 8), indicating that nuclear IκBβ maintained IκBα-insensitive NF-κB·DNA binding activity in HIV-infected cells. Supershift analysis revealed that similar NF-κB·DNA binding complexes were present before and after IκBβ immunodepletion in PMA (Fig 24C, lanes 1-4 and 9-12) and TNFα (Fig 24C, lanes 5-8 and 13-16) stimulated U9-IIIB (Fig 24C) and PLB-IIIB (data not shown) cells, arguing against the specific immunodepletion of an IκBα insensitive NF-κB complex.

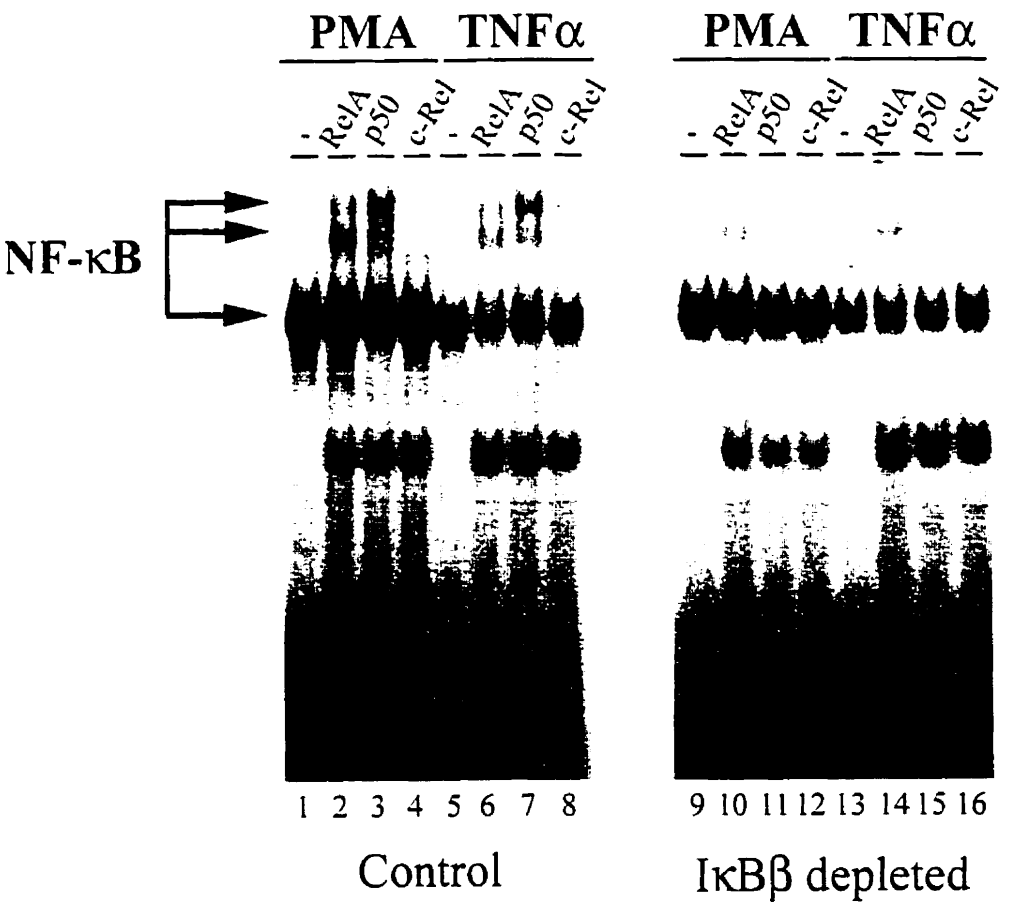
IκBβ co-expression increases NF-κB transcriptional activation. Next, a series of co-transfection experiments were performed to determine whether IκBβ co-expression affected NF-κB mediated transcription and to examine whether IκBβ blocked the inhibitory effect of IκBα. 293 cells were transfected with an NF-κB driven CAT reporter plasmid and empty vector, or reporter plasmid and pSVK3-IκBα, or reporter plasmid and both pSVK3-IκBα and pSVK3-IκBβ expression plasmids. PMA (50 ng/ml) was added for 0, 8, 16 or 24h and transactivation was assessed by comparing CAT activity. PMA stimulated cells

Figure 24. Depletion of IκBβ containing complexes sensitizes HIV-1 nuclear extracts to dissociation by IκBα. (**A**) DNA binding activity in U9-IIIB cells stimulated with PMA for 0, 2 or 18h and depleted for IκBβ by immunoprecipitation (IκBβ IP, lanes 9-15) were compared to undepleted nuclear extracts (NE, lanes 1-6). Extracts were incubated with labeled probe only (lanes 1, 3, 5, 7 9, 11, 13) or with labeled probe and GST-IκBα (lanes 2, 4, 6, 8, 10, 12 and 14). Immunoprecipitation with normal rabbit serum was used as a control for specificity (lanes 7 and 8). (**B**) PLB-IIIB NF-κB DNA binding activity in cells stimulated with PMA for 0 or 2h and depleted for IκBβ (IκBβ IP, lanes 1-4) was compared to undepleted similarly treated nuclear extracts (NE, lanes 5-8). Extracts were incubated with only labeled probe (lanes 1, 3, 5 and 7) or with labeled probe and GST-IκBα (lanes 2, 4, 6 and 8). (**C**) NF-κB subunit composition of U9-IIIB nuclear extracts stimulated with PMA or TNFα for 18h and immunodepleted of IκBβ (lanes 9-16) was compared to undepleted control extracts (lanes 1-8) by supershift analysis. RelA (lanes 2, 6, 10 and 14), p50 (lanes 3, 7, 11, 15) or c-Rel (lanes 4, 8, 12, 16) antibodies were utilized to identify the NF-κB DNA binding subunits present.


A
NE
IκBβ IP
PMA (h)
- 2 18 2 - 2 18 18
GST-IκBα
- + - + - + - + - + - + - + -
NF-κB
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15


B
NE
IκBβ IP
PMA (h)
- 2 - 2
GST-IκBα
- + - + - + - +
NF-κB
1 2 3 4 5 6 7 8

C


PMA
TNFα
PMA
TNFα
-
RelA
p50
c-Rel
-
RelA
p50
c-Rel
-
RelA
p50
c-Rel
-
RelA
p50
c-Rel
NF-κB
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16
Control
IκBβ depleted

transfected with both IκBα and IκBβ, partially alleviated the IκBα mediated repression of transcription (Fig. 25, compare 24h IκBα, IκBα & IκBβ and pSVK3 levels) and exhibited a 50% increase in NF-κB dependent expression compared to cells transfected with IκBα alone (Fig. 25). This result indicated that IκBβ expression partially reversed the inhibitory effects of IκBα and increased NF-κB mediated transcription.

Turnover of IκBβ isoforms. The regulation of IκBβ protein turnover was next investigated. Antibodies recognizing the 43-kDa isoform or both the 43- and 41-kDa isoforms were used to analyze the rate of IκBβ basal turnover and stimulus induced degradation. U937 and U9-IIIB cells were treated with cycloheximide (CHX) alone, or different inducers for 0, 2, 6 or 8h. The 43-kDa isoform of IκBβ exhibited faster constitutive turnover in HIV-infected U9-IIIB cells (compare Fig 26A, 26C, 26B and 26D, lanes 1-4) and also degraded more quickly following IL-1β (compare Fig 26A, 26C, 26B and 26D, lanes 9-12) or PMA (compare Fig 26A and 26D, lanes 5-8) stimulation. TNFα stimulation led to rapid degradation of 43-kDa IκBβ in both infected and uninfected cells (Fig 26B and 26C, lanes 5-8). The faster migrating 41-kDa form was not degraded by stimuli in U9-IIIB or U937 cells (Fig 26C and 26D respectively, lower band). Thus the dynamic state of degradation and resynthesis of the 43-kDa form of IκBβ may result in the continuous production of hypophosphorylated IκBβ, the form previously shown to shield DNA bound NF–κB from the effects of IκBα (288,415).

Constitutive activation of the IKK complex in HIV-infected cells. Since IκBα (113) and IκBβ turnover are increased in HIV-infected cells, we sought to determine whether an IκB kinase was constitutively active in HIV-infected cells. Using an antibody that recognizes the phosphoserine 32 of IκBα, HIV-infected U9-IIIB and PLB-IIIB cells but not their uninfected counterparts contained high levels of phosphorylated IκBα in the presence or absence of inducer (Fig 27A top panel, lanes 4-6 and 10-12). Stimulation of

Figure 25. IκBβ protects NF-κB transcriptional activity from inhibition by IκBα. 293 cells were transfected with an NF-κB CAT (7μg) reporter construct and pSVK3 control vector (8 μg) or pSVK3-IκBα (4μg) and/or with pSVK3-IκBβ (4μg) and stimulated with PMA (50 ng/ml) for 0, 8, 16 or 24h. Equal amounts of protein were assayed for CAT activity. Results are the average of a minimum of 3 experiments ± standard deviation.

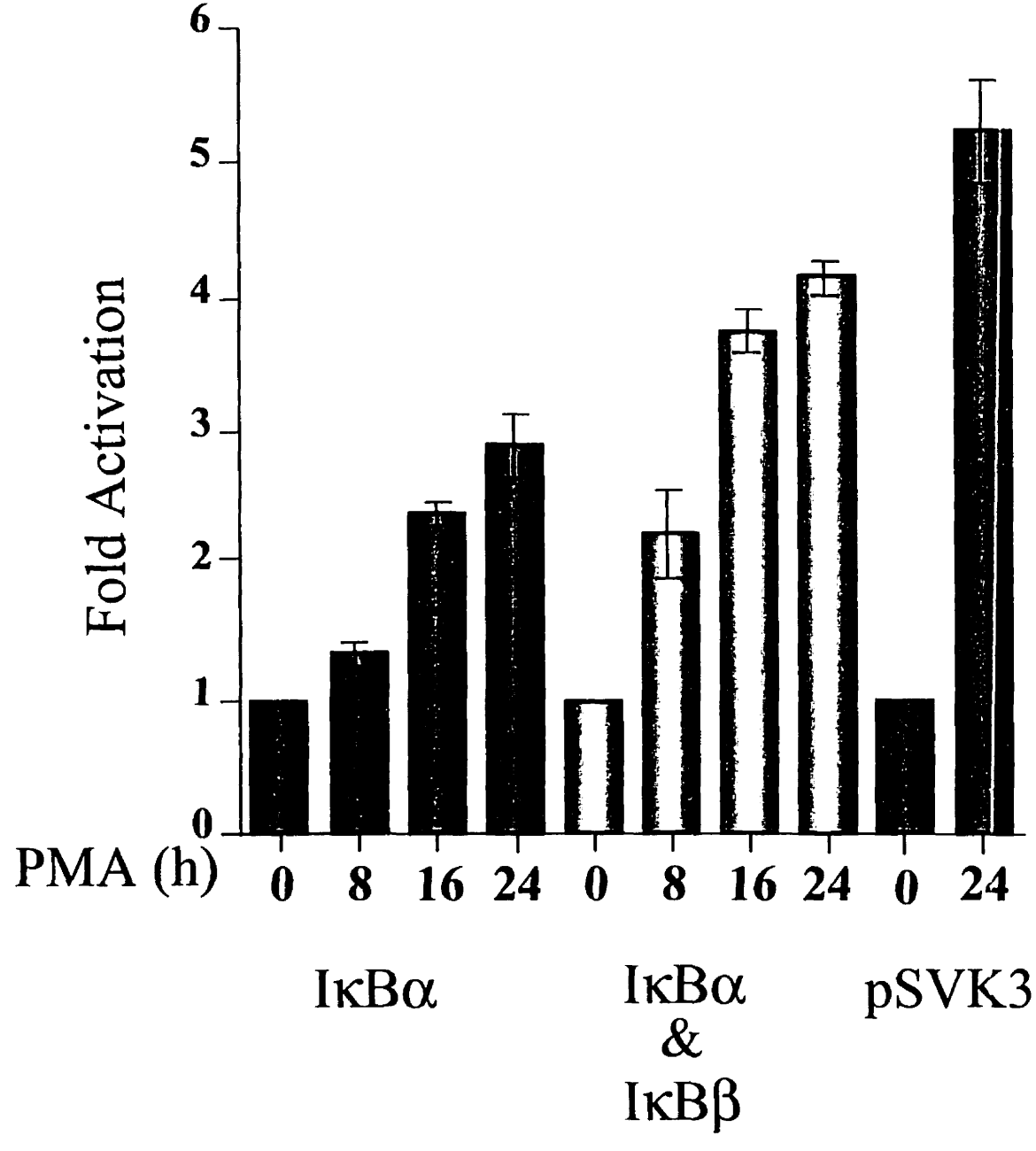
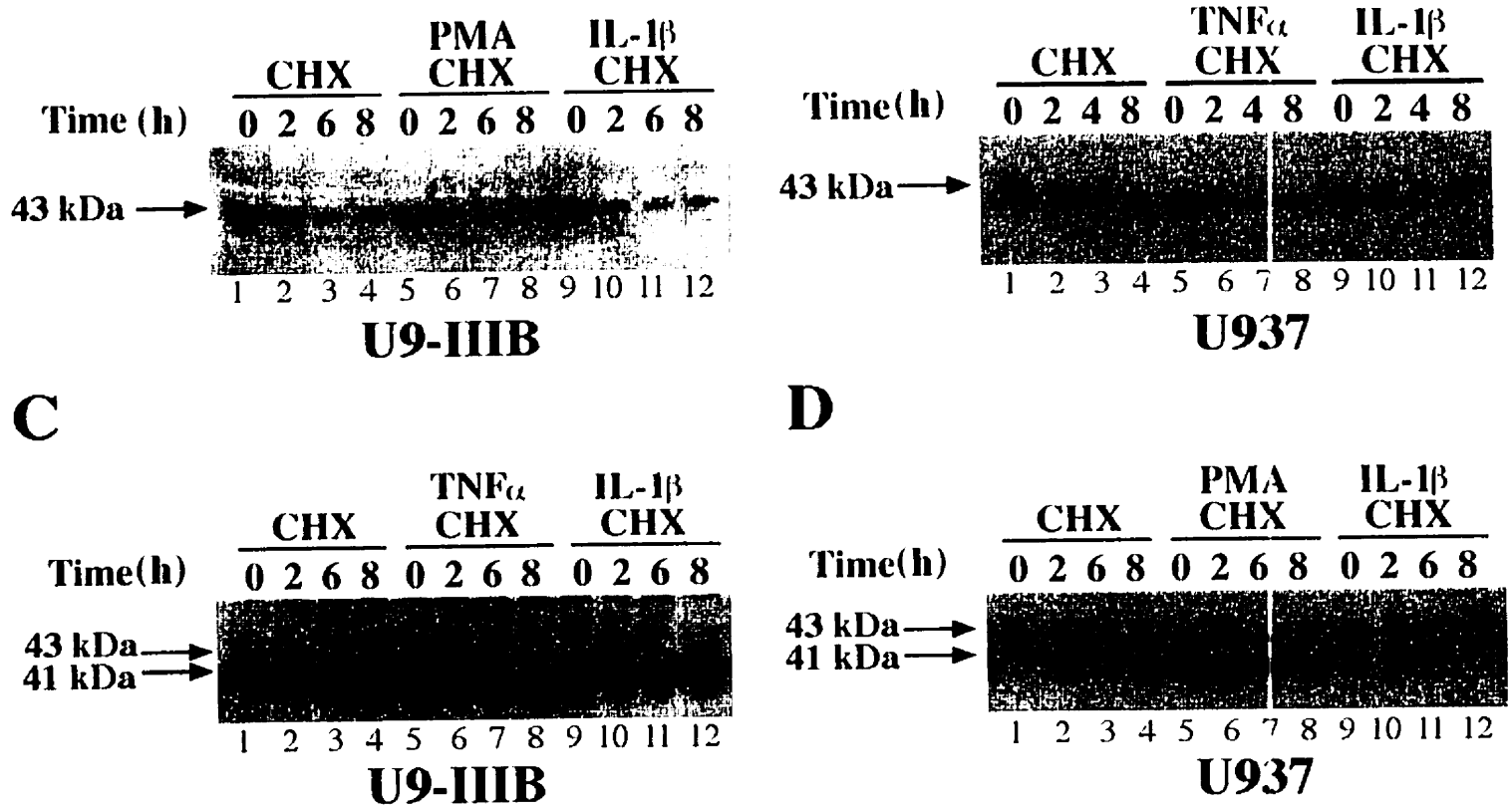

Fold Activation
6
5
4
3
2
1
0
PMA (h)
0 8 16 24 0 8 16 24 0 24
IκBα
IκBα & IκBβ
pSVK3

Figure 26. IκBβ turnover is increased in HIV-1 infected cells. (**A**) U9-IIIB cells were incubated with cycloheximide alone, cycloheximide and PMA (lanes 5-8) or cycloheximide (CHX) and IL-1β (lanes 9-12) for 0, 2, 6 or 8h. Whole cell extracts were separated by SDS-PAGE, transferred to nitrocellulose and probed with an IκBβ antibody which recognizes only the 43-kDa isoform. (**B**) U937 cells were incubated with CHX alone, CHX and TNFα or CHX and IL-1β for 0, 2 , 6 or 8h. Whole cell extracts were immunoblotted with anti-IκBβ (43-kDa isoform). (**C**) U9-IIIB cells were treated as described in B, immunoblotted and probed with an antibody that recognizes both the 43- and 41-kDa IκBβ isoforms. (**D**) U937 were treated as described in (**A**), immunoblotted and probed with an antibody that recognizes both isoforms of IκBβ.

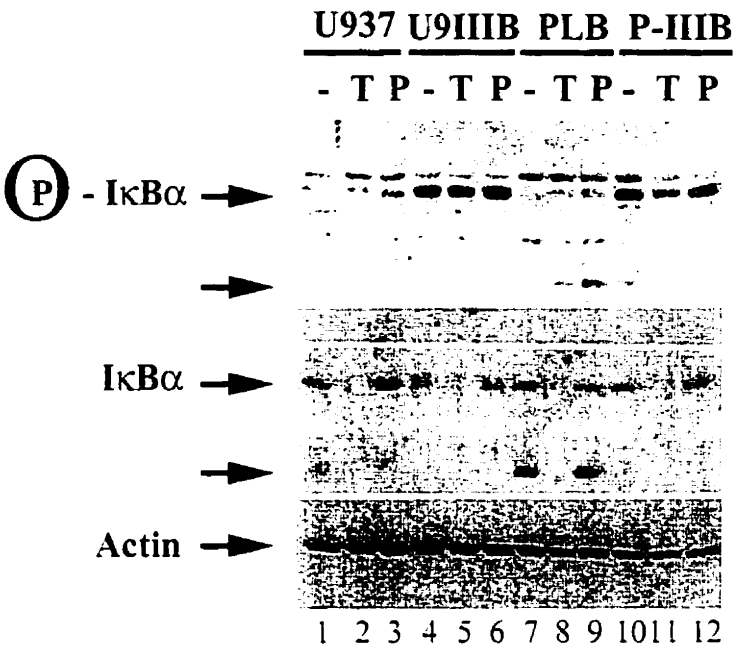

A
CHX
PMA CHX
IL-1β CHX
Time (h)
0 2 6 8 0 2 6 8 0 2 6 8
43 kDa
1 2 3 4 5 6 7 8 9 10 11 12
U9-IIIB
B
CHX
TNFα CHX
IL-1β CHX
Time(h)
0 2 4 8 0 2 4 8 0 2 4 8
43 kDa
1 2 3 4 5 6 7 8 9 10 11 12
U937
C
CHX
TNFα CHX
IL-1β CHX
Time(h)
0 2 6 8 0 2 6 8 0 2 6 8
43 kDa
41 kDa
1 2 3 4 5 6 7 8 9 10 11 12
U9-IIIB
D
CHX
PMA CHX
IL-1β CHX
Time(h)
0 2 6 8 0 2 6 8 0 2 6 8
43 kDa
41 kDa
1 2 3 4 5 6 7 8 9 10 11 12
U937

uninfected cells with TNFα or PMA for 10 min resulted in the appearance of phosphorylated IκBα in U937 (Fig 27A top panel, lanes 2 and 3) and PLB-985 (Fig 27A, lanes 8 and 9) cells. This blot was reprobed with monoclonal IκBα antibody (Fig 27A, middle panel) to confirm IκBα turnover. As expected, TNFα stimulation led to degradation of IκBα in all cell lines (Fig. 27A, middle panel, lanes 2, 5, 8 and 11). IκBα levels in PMA stimulated cells were not reduced (Fig. 27A, middle panel, lanes 3, 6, 9 and 12) although phosphorylated IκBα was detected, reflecting the longer kinetics of PMA induced IκBα degradation. As previously described, an IκBα immunoreactive band in PLB-985 cells of approximately 30-kDa in size was also detected (113). The 30-kDa IκBα was also recognized by the phosphoserine specific IκBα antibody (Fig. 27A, top panel, lower arrow) and degraded similarly to IκBα (Fig. 27A, middle panel), suggesting that the regulation of the 30-kDa form may be similar to that of full length IκBα.

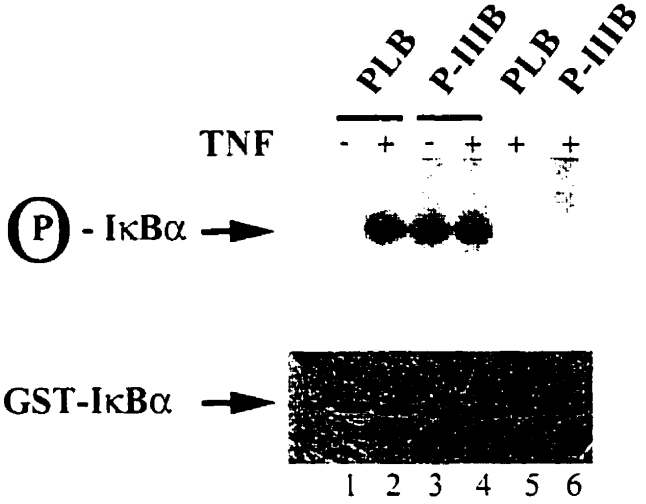
Given the crucial role of the IκB kinase complex (IKK) in the activation cascade of NF-κB, the possibility of constitutive IKK activity in HIV-infected cells was also examined. PLB-985 and PLB-IIIB cells were stimulated with TNFα for 15 minutes, extracts were immunoprecipitated with anti-IKK antibody and immunoprecipitates analysed for the ability to phosphorylate N-terminal IκBα (1-55) *in vitro*. PLB-985 cells exhibited little or no IKK activity (Fig 27B, lane 1) unless stimulated with TNFα (Fig 27B, lane 2), whereas HIV-infected cells displayed IKK activity with or without TNFα stimulation (Fig 27B, lanes 3-4). This activity was specific since immunoprecipitation with normal rabbit serum did not result in detectable kinase activity (TNFα stimulated PLB-985, Fig 27B, lane 5) and mutated IκBα substrate (1-55, S32/36A) was not phosphorylated (TNFα stimulated PLB-IIIB, Fig 27B, lane 6). Coomassie blue staining revealed that equal amounts of IκBα substrate were used in each reaction (Fig 27B, bottom panel). Similarly, IKK activity was inducible by TNFα in U937 cells (Fig 27C, lanes 1-3) but was constitutively activated in U9-IIIB cells (Fig 27C, lanes 4-6).

Figure 27. The IκB kinase complex is constitutively active in HIV-1 infected cells. (**A**) Cells were untreated, treated with TNFα (T) or PMA (P) for 10 min. Whole cell extracts were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted using an IκBα antibody that recognizes serine 32 phosphorylated IκBα (upper panel). The blot was reprobed using a monoclonal IκBα antibody (middle panel) and then reprobed with an actin antibody as a control for equal loading. (**B**) PLB-985 (PLB, lanes 1, 2 and 5) and PLB-IIIB cells (P-IIIB, lanes 3, 4, and 6) were treated with TNFα for 0 or 15 min and assayed for IKK using GST-IκBα (1-55) as substrate. Specificity was confirmed using normal rabbit serum as the immunoprecipitating antibody (lane 5) and by using GST-IκBα (1-55; S32/36A) as substrate (lane 6). Coomassie blue staining of the gel (bottom panel) reveals that equal amounts of recombinant protein were used in each reaction. (**C**) U937 (lanes 1-3 and 7) and U9-IIIB (4-6 and 8) cells were stimulated with TNFα for 1 or 12h and analyzed for IKK activity using GST-IκBα (1-55) as substrate. Specificity was confirmed using normal rabbit serum as the immunoprecipitating antibody (lane 7) and by using GST-IκBα (1-55; S32/36A) as substrate (lane 8).

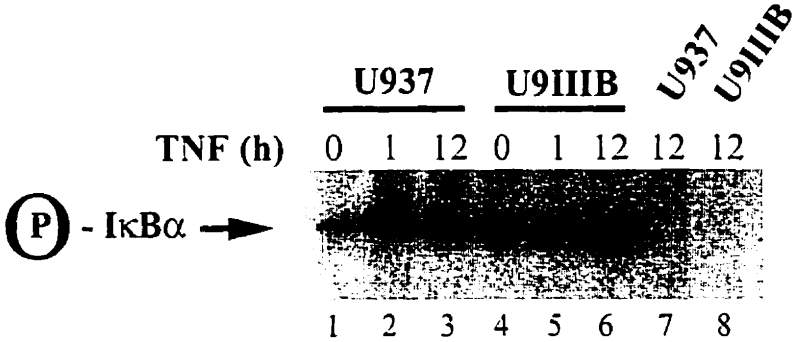
A


U937 U9IIIB PLB P-IIIB
- T P - T P - T P - T P
P - IκBα
IκBα
Actin
1 2 3 4 5 6 7 8 9 10 11 12

B


PLB
P-IIIB
PLB
P-IIIB
TNF
- + - + + +
P - IκBα
GST-IκBα
1 2 3 4 5 6

C


U937
U9IIIB
U937
U9IIIB
TNF (h)
0 1 12 0 1 12 12 12
P - IκBα
1 2 3 4 5 6 7 8

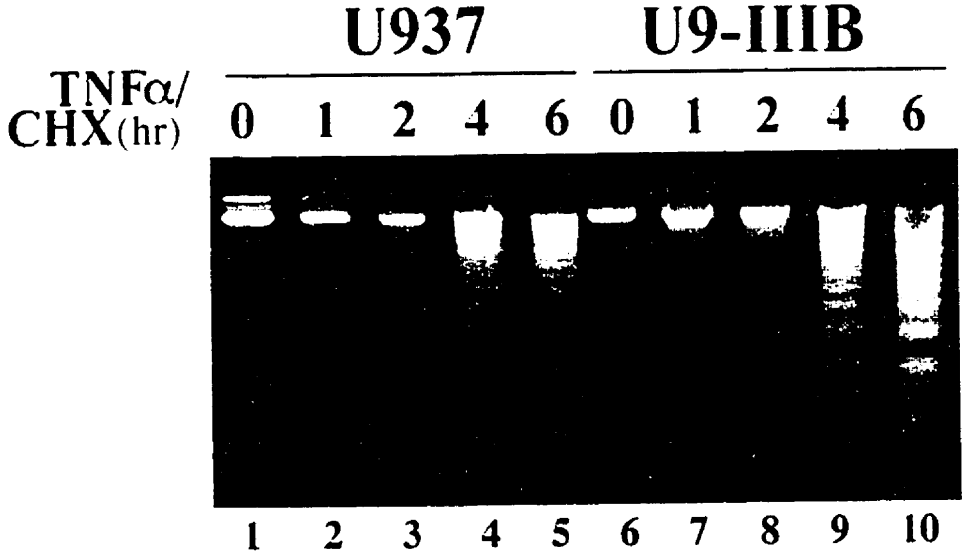
Chapter V

NF-κB PROTECTS HIV-1 INFECTED MYELOID CELLS FROM APOPTOSIS

Increased apoptosis in HIV-infected U937 cells. Experiments presented in this chapter derive from three observations: 1) chronic HIV-1 infection of myeloid cells results in constitutive NF-κB DNA binding activity (25,370,372,373) as a result of increased IκBα degradation and IκBβ mediated protection of DNA binding complexes; NF-κB has a bipotential role in apoptotic signalling, protective in some cells (47,437,444) pro-apoptotic in others (9,213,262); and 3) HIV-1 infection is associated with virus induced apoptosis in susceptible cells. We therefore asked whether perturbation of NF-κB activity in U937/U9-IIIB or PLB-985/PLB-IIIB cells resulted in altered apoptotic signalling. U937 and U9-IIIB cells were incubated in the presence of 10 ng/ml TNFα and 100 μg/ml cycloheximide (CHX) for 0 1, 2, 4 or 6h and analysed for DNA fragmentation. Figure 28 illustrates that U9-IIIB cells exhibited increased sensitivity to TNFα, undergoing detectable DNA fragmentation as early as 1-2h after TNFα treatment (Fig. 28, lanes 7,8) compared to uninfected U937 which do not show visible signs of apoptosis until 4h (Fig. 28, lane 4). Increasing the concentration of TNFα induced a more rapid induction of apoptosis in both cell types (data not shown). Similar results were obtained using promonocytic PLB-985 cells and HIV-infected PLB-IIIB cells. These cells were treated with TNFα (20 ng/ml) and CHX (50 μg/ml) for 0-5h. Infected cells exhibited DNA laddering after 1h while uninfected cells underwent fragmentation beginning at 2h (data not shown).

Relationship between NF-κB levels and apoptosis. To assess the involvement of NF-κB in apoptotic signalling, U937 and U9-IIIB cells were pretreated with the antioxidant N-acetyl-cysteine (NAC; 50 mM) for 1h and then treated with TNFα (20 ng/ml) and CHX (50 μg/ml) for 2h; NAC inhibits NF-κB activation by blocking IκB phosphorylation (254,391,405). The level of TNFα/CHX induced apoptosis was increased in both U937 and U9-IIIB cells following NAC pretreatment as quantified by the TUNEL assay (Fig. 29A and 29B). TNFα/CHX induced approximately 30% apoptosis in

Figure 28. Chronically HIV infected U9-IIIB cells undergo apoptosis in response to TNFα/CHX more rapidly than uninfected U937 cells. Cells were treated with the protein synthesis inhibitor cycloheximide (CHX 100 μg/ml) and tumor necrosis factor α (TNFα 10 ng/ml) for 0, 1, 2 , 4 or 6h. 2 x 10^6 cells were lysed and analyzed for DNA fragmentation by electrophoresis and visualized by ethidium bromide staining. Fragmentation was visible after 1-2h in U9-IIIB (lanes 7-8) cells but not until 4h in U937 cells (lane 4).


U937
U9-IIIB
TNFα/
CHX(hr)
0 1 2 4 6 0 1 2 4 6
1 2 3 4 5 6 7 8 9 10

U937 (Fig. 29A and 29C) and this value was increased to 50% by NAC pretreatment. TNFα/CHX treatment induced 35 to 70% apoptosis in U9-IIIB cells (Fig. 29B and 29D); NAC pretreatment resulted in a consistent 20-40% increase in apoptotic cells (Fig. 29B). Interestingly, the combination of NAC/CHX induced observable apoptosis in U9-IIIB whereas U937 cells were only marginally affected. In addition, CHX alone caused a low level of apoptosis in U9-IIIB cells (~10%), suggesting that a labile factor may be important for cell survival in HIV-infected cells. Again, similar results were obtained using PLB-IIIB cells (Table 3).

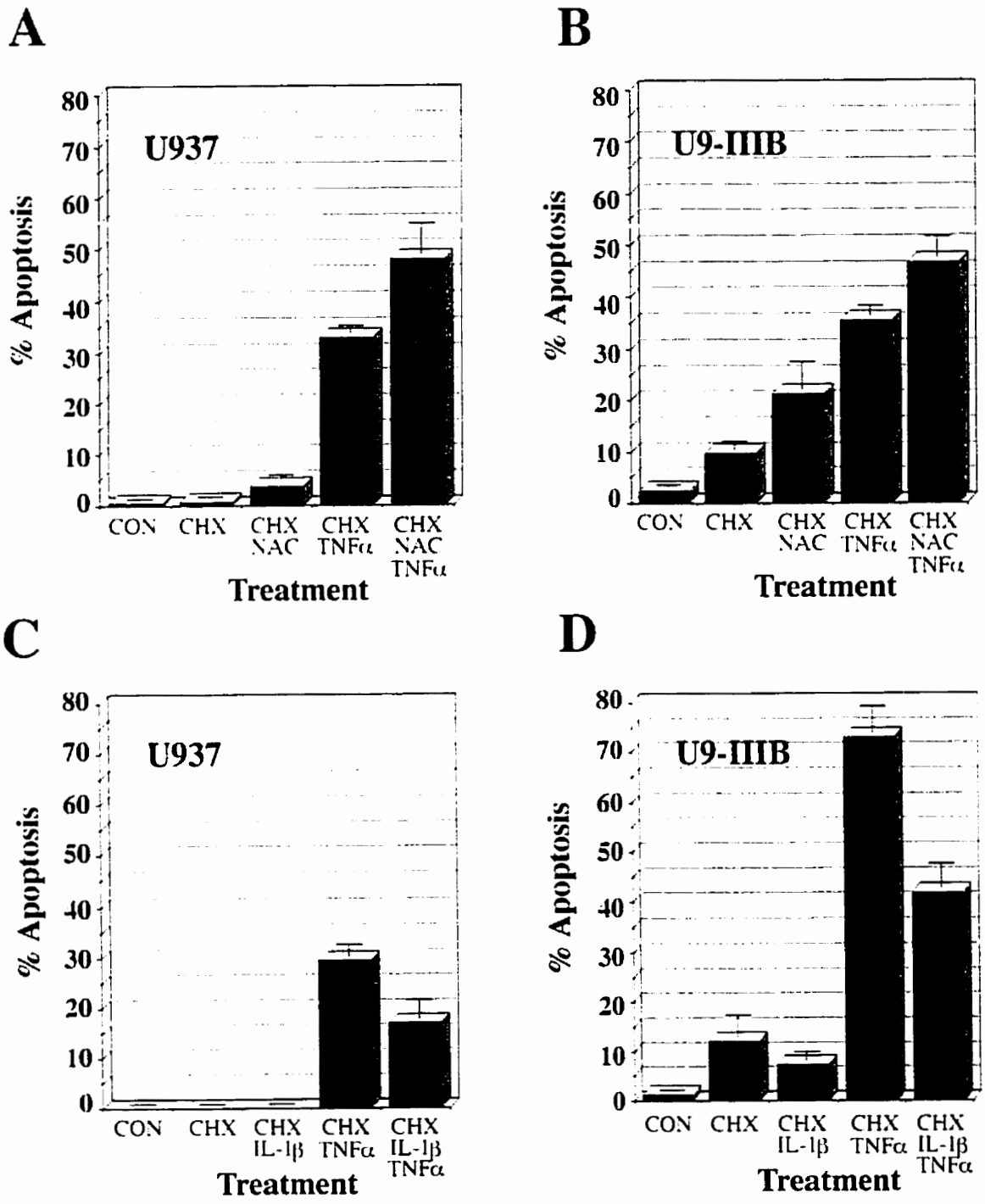
Table 3. Effect of NF-κB activation on TNFα/CHX induced apoptosis in PLB-IIIB cells.

Condition	Percent Apoptosis
Untreated	1.3
Cycloheximide	11.9 ± 0.54
NAC + Cycloheximide	33.5 ± 2.2
IL-1β + Cycloheximide	6.9 ± 0.57
TNFα + Cycloheximide	39.8 ± 5.2
NAC + TNFα + Cycloheximide	57.5 ± 1.7
IL-1β + TNFα + Cycloheximide	27.9 ± 3.4

PLB-IIIB cells were pretreated with IL-1β (10 ng/ml) for 5h or NAC (50 mM) for 1h and were subsequently incubated with TNFα(20 ng/ml)/CHX (50 μg/ml) for 2h. The number of apoptotic cells was quantified by TUNEL assay. Values are the result of an experiment performed in triplicate.

To determine if NF-κB preactivation would decrease apoptosis, U937 and U9-IIIB cells were stimulated with IL-1β (5 ng/ml), a non-cytotoxic inducer of NF-κB, for 5h followed by a 2h incubation with TNFα (20 ng/ml) and CHX (50 μg/ml). In both cell types, addition of IL-1β reduced both TNFα/CHX induced apoptosis by approximately 40%, as well as the low level of CHX-induced apoptosis in HIV-infected cells (Fig. 29C and 29D), again suggesting that a labile protein, induced by constitutive NF-κB, maintained cell

Figure 29. Inhibition of NF-κB increases - while preactivation of NF-κB decreases - TNFα/CHX induced apoptosis. U937 (**A**) and U9-IIIB (**B**) cells were pretreated with or without the antioxidant N-acetyl cysteine (NAC 50 mM) for 1h or U937 (**C**) and U9-IIIB (**D**) cells were pretreated with or without interleukin 1β (IL-1β 5 ng/ml) for 5h. Cells were subsequently treated with cycloheximide (CHX 50 μg/ml) and/or tumor necrosis factor α (TNFα 20 ng/ml) for 2h. Apoptotic cells were measured by TUNEL assay and the total cell numbers determined by Hoechst staining. Percent apoptotic cells were calculated with the results plotted for an experiment carried out in triplicate.


A
U937
% Apoptosis
80
70
60
50
40
30
20
10
0
CON
CHX
CHX NAC
CHX TNFα
CHX NAC TNFα
Treatment
B
U9-IIIB
% Apoptosis
80
70
60
50
40
30
20
10
0
CON
CHX
CHX NAC
CHX TNFα
CHX NAC TNFα
Treatment
C
U937
% Apoptosis
80
70
60
50
40
30
20
10
0
CON
CHX
CHX IL-1β
CHX TNFα
CHX IL-1β TNFα
Treatment
D
U9-IIIB
% Apoptosis
80
70
60
50
40
30
20
10
0
CON
CHX
CHX IL-1β
CHX TNFα
CHX IL-1β TNFα
Treatment

survival. Figure 30 illustrates U937 cells positive for apoptosis by TUNEL assay (right panels) within the total cell population (left panels) induced by the treatments described in Figure 29.

NF-κB·DNA binding activity in TNFα, IL-1β and NAC treated nuclear extracts was assessed by EMSA. Figure 31A illustrates that both TNFα and IL-1β induced NF-κB·DNA binding activity in U937 (Fig. 31A, lane 4, 5 and 7) and U9-IIIB cells (Fig. 31A, lanes 11, 12 and 14) and this level of DNA binding was inhibited by NAC preincubation (Fig. 31A, lanes 6 and 13 respectively). U9-IIIB cells exhibited constitutive NF-κB·DNA binding activity (Fig. 31A, lane 8) which was abolished by NAC treatment (Fig. 31A, lane 10); specificity of the complex was confirmed by competition with a 125 molar excess of unlabeled competitor DNA (Fig. 31A, lane 15). Figure 31B illustrates that TNFα/CHX induced IκBα degradation in both U937 and U9-IIIB cells (Fig. 31B, lanes 5, 7 and 12, 14 respectively) and this degradation was inhibited by NAC pretreatment (Fig. 31B, lanes 6 and 13). PLB-IIIB cells showed a similar pattern of IκBα degradation in response to the conditions described above (data not shown).

TNFα alone also induces DNA fragmentation in U937 and U9-IIIB cells, with the latter exhibiting signs of DNA fragmentation earlier than non-infected cells. U937 and U9-IIIB cells were incubated with TNFα (20 ng/ml) and/or NAC (30 mM) for 0 to 48h, and in all cases, NAC was added to cells 1h prior to TNFα addition. U937 cells did not undergo apoptosis when incubated with NAC (Fig. 32A, lane 2) but underwent DNA fragmentation when incubated with TNFα (Fig. 32A, lane 3) or TNFα and NAC (Fig. 32A, lane 4). In contrast, U9-IIIB cells were sensitive to all three treatments undergoing DNA fragmentation by 8h after NAC (Fig. 32A, lane 6), TNFα (Fig. 32A, lane 7) or TNFα plus NAC (Fig. 32A, lane 8) addition. Electrophoretic mobility shift analyses (EMSA) of nuclear extracts from TNFα and/or NAC treated cells confirmed that NAC inhibited both

Figure 30. TUNEL analysis of U937 cells pretreated with NAC or IL-1β followed by TNFα/CHX treatment. U937 cells were treated with NF-κB inducers or inhibitors, fixed, analyzed for TUNEL positivity and photographed (magnification 200X). **A**) Cells were pretreated with N-acetyl cysteine (NAC 50 mM) for 1h followed by cycloheximide (CHX 50 μg/ml) for 2h, **B**) cells were treated with tumor necrosis factor α (TNFα 20 ng/ml) and CHX (50 μg/ml) for 2h. **C**) cells were pretreated with NAC (50 mM) for 1h and incubated with TNFα (20 ng/ml) and CHX (50 μg/ml) for 2h. **D**) Cells were pretreated with interleukin-1β (IL-1β 5 ng/ml) for 5h and stimulated with TNFα (20 ng/ml) and CHX (50 μg/ml) for 2h. Total cells (on the left) were stained with Hoechst 33342 while apoptotic cells (on the right) were detected using the TUNEL assay (Boehringer Mannheim) as described in Materials and Methods.

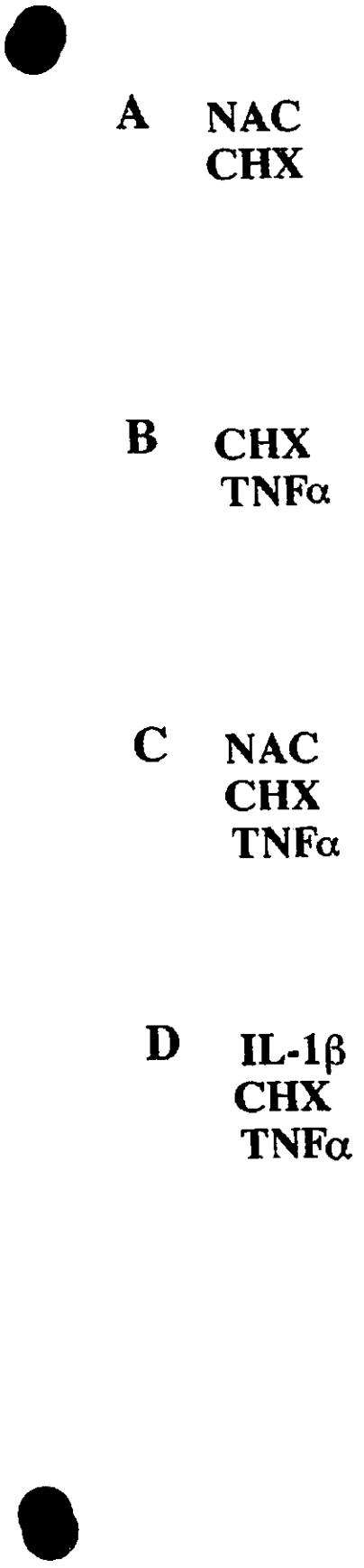
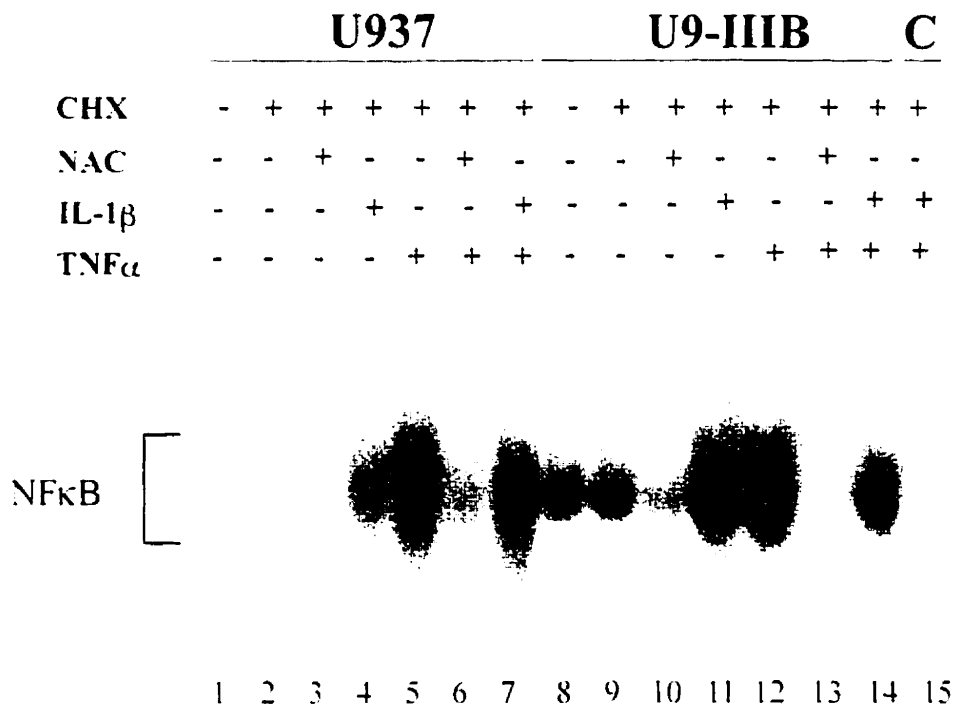
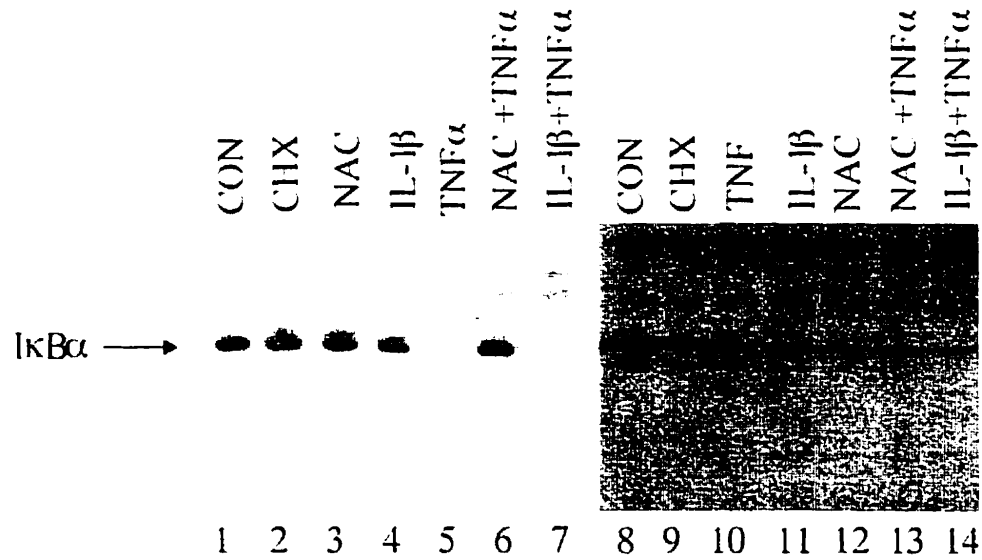

A
NAC
CHX
B
CHX
TNFα
C
NAC
CHX
TNFα
D
IL-1β
CHX
TNFα

Figure 31. Analysis of NF-κB·DNA binding activity and IκBα protein levels. **(A)** Nuclear extracts from samples treated as in Figure 29 were analyzed for NF-κB activity using a ^{32}P labeled probe containing two NF-κB sites from the IFN-β promoter. U9-IIIB cells exhibit constitutive NF-κB DNA binding activity (lane 8) that is increased by TNFα and IL-1β (lanes 11, 12 and 14). U937 cells exhibit binding activity upon TNFα and/or IL-1β stimulation (lanes 4, 5 and 7). NAC pretreatment inhibited both the constitutive binding activity in U9-IIIB cells (lane 10) as well as TNFα induced NF-κB binding (lanes 6 and 13). **(B)** Whole cell extracts were made from cells treated as described in Figure 29 and protein (30 μg) was separated by SDS-PAGE and blotted for IκBα protein. IκBα is degraded by TNFα in both U937 (lanes 5 and 7) and U9-IIIB cells (lanes 12 and 14) and this degradation is blocked by pretreatment with NAC (lanes 6 and 13 respectively). Equal amounts of protein were confirmed by actin staining (data not shown).

A


U937
U9-IIIB
C
CHX - + + + + + + - + + + + + + +
NAC - - + - - + - - - + - - + - -
IL-1β - - - + - - + - - - + - - + +
TNFα - - - - + + + - - - - + + + +
NFκB
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

B

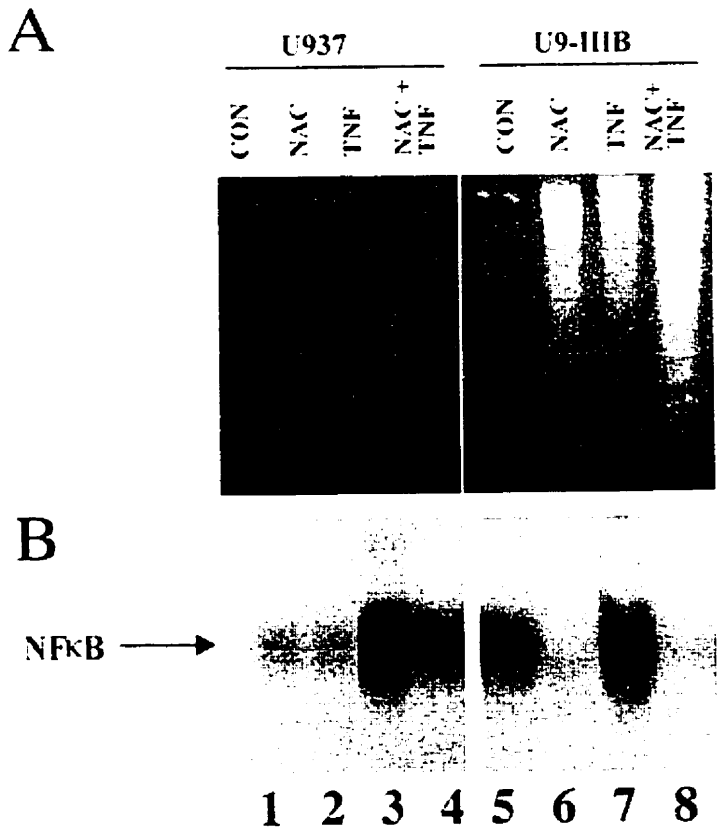

CON
CHX
NAC
IL-1β
TNFα
NAC +TNFα
IL-1β+TNFα
CON
CHX
TNF
IL-1β
NAC
NAC +TNFα
IL-1β+TNFα
IκBα
1 2 3 4 5 6 7 8 9 10 11 12 13 14

TNFα induced NF-κB binding and constitutive NF-κB in HIV-infected cells (Fig. 32B). NF-κB binding was detected in TNFα induced extracts from U937 and U9-IIIB cells (Fig. 32B, lanes 3 and 7 respectively) but was reduced by NAC preincubation (Fig. 32B, lanes 4 and 8). In addition, constitutive NF-κB in U9-IIIB cells (Fig. 32B, lane 5) was blocked by NAC (Fig. 32B, lane 6).

Bcl-2/Bax expression in HIV-infected cells. These results suggest that NF-κB may regulate genes important for protecting cells from apoptosis. One candidate gene is bcl-2; Bcl-2 protein antagonizes apoptosis and is known to be NF-κB regulated (32). To analyze Bcl-2 expression, U937 and U9-IIIB cells were treated with NAC (30 mM), TNFα (20 ng/ml) or TNFα/NAC for 0, 8, 16, 24 or 48h and whole cell extracts (50 μg) were examined for Bcl-2 expression levels. NAC had little effect on Bcl-2 levels in U937 cells but resulted in a decrease in Bcl-2 protein level in U9-IIIB cells (data not shown). TNFα/NAC treatment caused a significant decrease in Bcl-2 protein with time in U9-IIIB cells (Fig. 33A, lanes 6-10) but only a slight decrease in U937 cells (Fig. 33A, lanes 1-5). Bcl-2 protein levels relative to actin levels are plotted as a function of time for TNFα/NAC treated cells.

Bcl-2 protein levels were approximately equal in U937 and HIV-infected U9-IIIB cells and therefore could not account for the difference in sensitivity of U937 and U9-IIIB cells to TNFα induced apoptosis. Previous studies demonstrated that HIV-1 Tat expression resulted in increased Bax expression, a pro-apoptotic member of the Bcl-2 family (383). To determine whether differential expression of Bax contributed to the differences in sensitivity to apoptosis in U9-IIIB cells, extracts were prepared from cells treated with NAC, TNFα or TNFα/NAC for 24h. U9-IIIB cells expressed approximately 2 to 3 fold higher levels of Bax compared to U937 cells (Fig. 33B, lanes 1 and 5). NAC treatment did not affect Bax in U937 cells or U9-IIIB cells (Fig. 33B, lanes 2 and 6) while TNFα

Figure 32. NAC treatment induces DNA fragmentation in U9-IIIB cells but not in U937 cells. (**A**) U937 and U9-IIIB cells were treated with N-acetyl cysteine (NAC 30mM) or tumor necrosis factor α (TNFα 20ng/ml) or both for 24h. Samples were collected and analyzed for DNA fragmentation. U937 cells undergo apoptosis in response to TNFα (lane 3) or TNFα plus NAC (lane 4). U9-IIIB cells are also sensitive to TNFα (lane 7) or TNFα plus NAC treatment (lane 8) but in addition exhibit DNA fragmentation when treated with only NAC (lane 6). (**B**) Nuclear extracts were made from U937 and U9-IIIB cells treated as above. NAC inhibits constitutive NF-κB activity in U9-IIIB cells (lane 6) as well as TNFα induced binding in both cell lines (lanes 4 and 8).


A
U937
U9-IIIB
CON
NAC
TNF
NAC + TNF
CON
NAC
TNF
NAC + TNF
B
NFκB
1 2 3 4 5 6 7 8

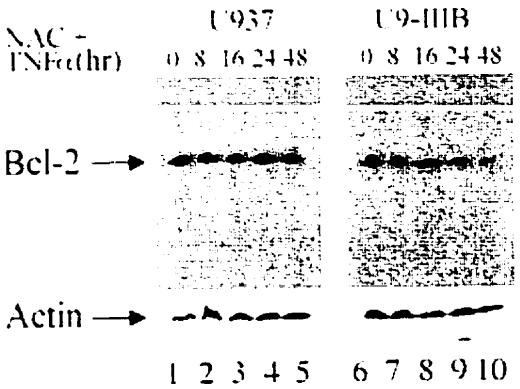
treatment increased Bax levels approximately 2-fold in U937 and U9-IIIB (Fig. 33B, lanes 3 and 7 respectively). NAC only partially inhibited TNFα induced increase in Bax protein in U937 and U9-IIIB cells (Fig. 33B, lanes 4 and 8), suggesting its activation by TNFα may in part be regulated by ROIs.

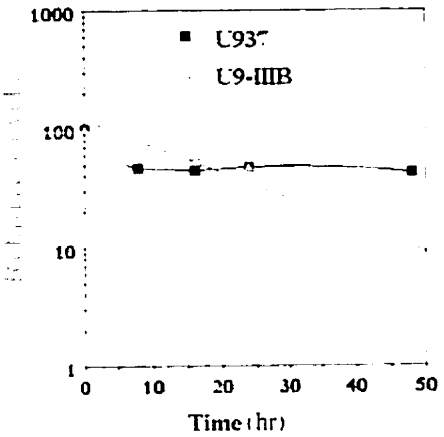
Induction of apoptosis in T cells expressing IκBα repressor mutants. The protective role of NF-κB in preventing TNFα induced apoptosis was verified in Jurkat T cells inducibly expressing non-degrading transdominant forms of IκBα (TD-IκB) under the control of a tetracycline responsive promoter (239). In this system doxycycline (Dox) addition induces high levels of TD-IκB which effectively block TNFα induced NF-κB activation (239). Control Jurkat cells (rtTA-Neo), and Jurkat cells expressing the IκB repressors (rtTA-IκB-2N and rtTA-IκB-2NΔ4, mutated at S32/36A) were incubated with or without Dox (1 μg/ml) for 16h followed by TNFα (20 ng/ml) treatment for 0-24h. As shown in Fig. 34, TNFα did not induce apoptosis in control rtTA-Neo cells either in the absence or presence of Dox (Fig. 34A, lanes 1-5 and 6-10). Similarly, no DNA fragmentation was observed in the absence of Dox in the rtTA-IκB-2N and rtTA-IκB-2NΔ4 cells (Fig. 34B and 34C, lanes 1-5). In contrast, rtTA-IκB-2N and rtTA-IκB-2NΔ4 cells displayed DNA fragmentation after Dox induction (expression of the IκB repressors) and TNFα addition (Fig. 34B and 34C, lanes 6-10), demonstrating an increased sensitivity to apoptosis in cells blocked for NF-κB dependent activity.

Dox-induction of TD-IκB repressors was verified by immunoblot analysis (Fig. 35A) and the subsequent inhibition of NF-κB·DNA binding activity was observed by EMSA (Fig. 35B). Addition of Dox to rtTA-2N and rtTA-2NΔ4 resulted in increased TD-IκB levels (Fig. 35A, lanes 5, 6, 8 and 9); interestingly, as previously described, activation of the 2NΔ4 mutant resulted in the inhibition of endogenous IκBα, as distinguished from 2NΔ4 IκBα using the MAD 10B antibody (19) (Fig. 35A, lanes 7-9). Furthermore, TNFα

Figure 33. Bcl-2 and Bax protein levels in U937 and U9-IIIB cells. U937 and U9-IIIB cells were treated with tumor necrosis factor α (TNFα 20 ng/ml) and N-acetyl cysteine (NAC 30 mM) for 0, 8, 16, 24 or 48h. Whole cell extracts were electrophoresed and blotted for Bcl-2 and β-actin protein levels. The ratio of Bcl-2 to β-actin was calculated and plotted on a semi-log scale. (**A**) Bcl-2 levels dramatically decrease in NAC/TNFα treated U9-IIIB cells while showing only a modest decrease in U937. (**B**) The 24h timepoints were analyzed for Bax levels by western blot analysis. U937 cells (left panel) express barely detectable levels of Bax protein which increases upon TNFα induction (lane 3). U9-IIIB cells (right panel) express a higher level of Bax protein (lane 5) that is further augmented by TNFα treatment (lane 7).

A


U937
U9-IIIB
NAC -
TNFα(hr)
0 8 16 24 48
0 8 16 24 48
Bcl-2
Actin
1 2 3 4 5
6 7 8 9 10


1000
100
10
1
U937
U9-IIIB
0
10
20
30
40
50
Time (hr)

B

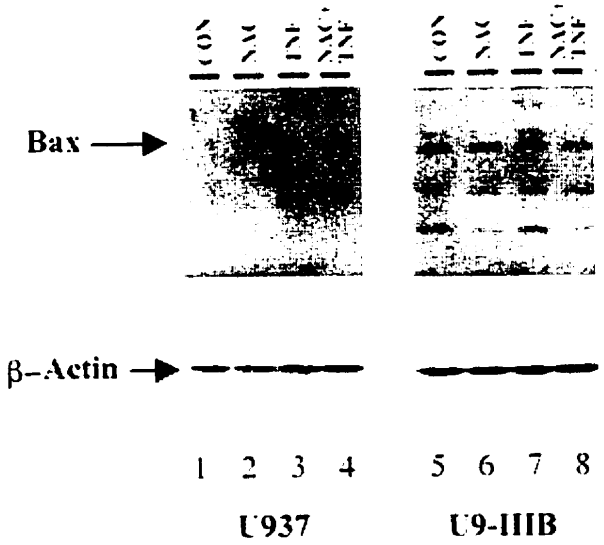
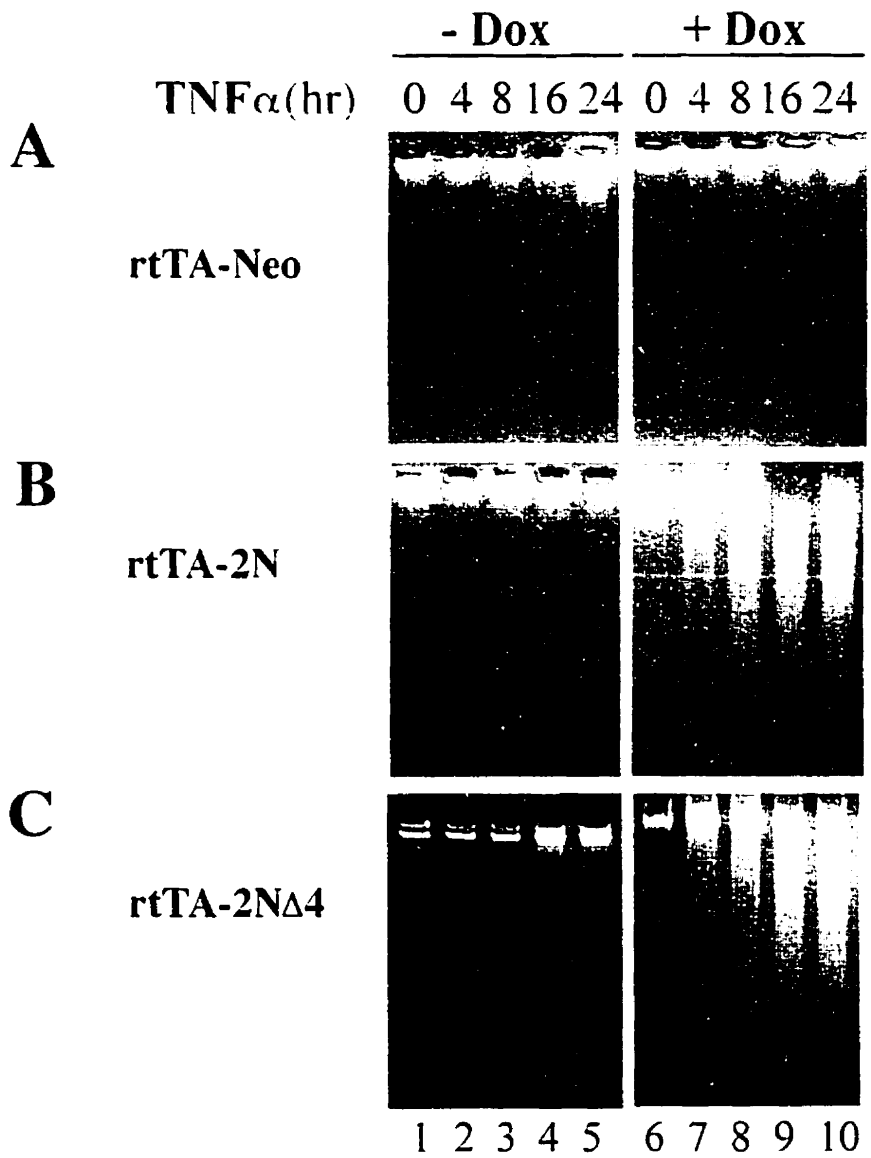

Bax
β–Actin
1 2 3 4
5 6 7 8
U937
U9-IIIB

Figure 34. TD-IκB expression induces sensitivity to TNFα signaled apoptosis. Jurkat T cells inducibly expressing IκBα mutated in two critical serine residues abolishing inducer mediated degradation, were treated with tumor necrosis factor α (TNFα 20 ng/ml) for 0, 4, 8, 16 or 24h. Transdominant IκBα was induced prior to TNFα treatment with doxycycline (Dox 1μg/ml) for 16h. TNFα treatment does not induce DNA fragmentation in control rtTA-Neo cells (**A**) but does in rtTA-2N (**B**) and rtTA-2NΔ4 (**C**) cells which express transdominant IκBα (compare lanes 1-5 and 6-10).


- Dox
+ Dox
TNFα(hr)
0 4 8 16 24 0 4 8 16 24
A
rtTA-Neo
B
rtTA-2N
C
rtTA-2NΔ4
1 2 3 4 5 6 7 8 9 10

stimulation did not affect TD-IκB (Fig. 35B, lanes 6 and 9). Nuclear extracts from these cells confirmed that NF-κB could be activated in all three cell lines in the absence of Dox (Fig. 35B, lanes 2, 6 and 10). Dox treatment for 24h led to the complete inhibition of TNFα induced binding activity in cells expressing TD-IκB (Fig. 35B, lanes 8 and 12) but not in control cells (Fig. 35B, lane 4). rtTA-2N (Fig. 35C, lanes 7-12) and rtTA-2NΔ4 (Fig. 35C, lanes 13-18) cells induced to express the TD-IκB repressors, also downregulated Bcl-2 protein levels upon prolonged TNFα treatment indicating that bcl-2 expression is controlled by NF-κB induction. Bcl-2 levels in control cells were not affected (Fig 35C, lanes 1-6) and equal protein was confirmed by actin staining (data not shown). rtTA-Jurkat cells were subsequently treated with combinations of NAC, CHX, and TNFα in the absence of TD-IκB induction to determine if inhibition of NF-κB activation by NAC rather than TD-IκB expression produced similar results. As shown in Table 4, TNFα/CHX treatment induced apoptosis which was further augmented by NAC pretreatment, as was described above for myeloid cells.

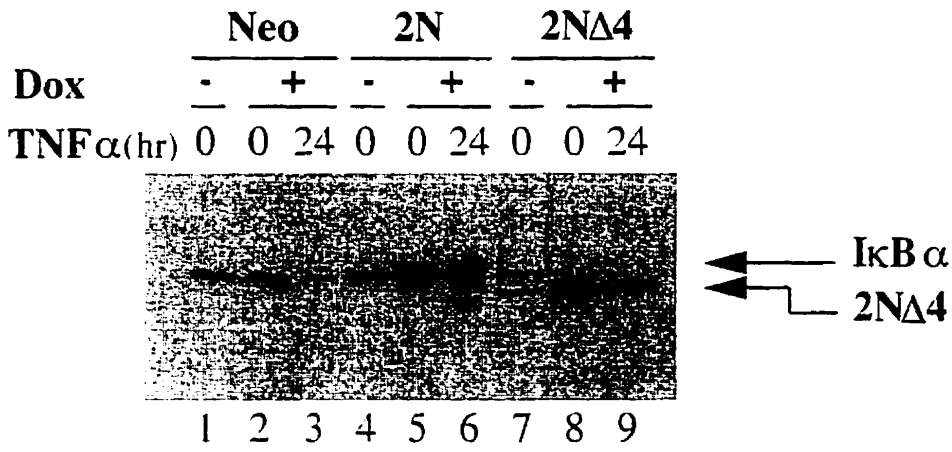
Table 4. NAC inhibition of TNFα induced NF-κB activation augments apoptosis in Jurkat cell lines.

Condition	rtTA-Neo	rtTA-2NΔ4
Control	0.52	8.2
Cycloheximide	14.1 ± 0.54	17.5 ± 1.8
NAC + Cycloheximide	14.4 ± 1.3	15.2 ± 1.2
TNFα + Cycloheximide	22.0 ± 1.2	30.6 ± 2.1
NAC+TNFα+Cycloheximide	54.5 ± 8.7	45.0 ± 4.5

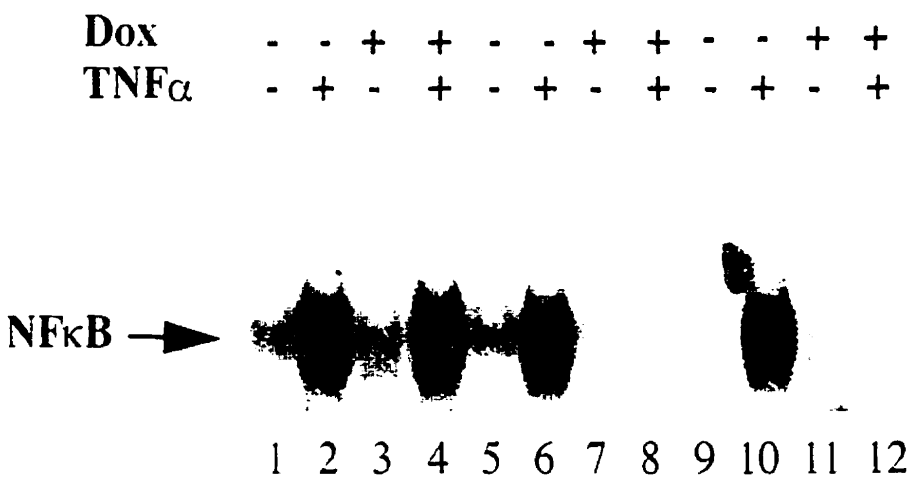
Jurkat rtTA-Neo and rtTA-2NΔ4 cells were pretreated with NAC (30 mM) for 1h and stimulated with TNFα (30 ng/ml) CHX (50 μg/ml) for 2h. Tunel assay was used to quantitate the number of apoptotic cells. Values are the average percent apoptosis of two experiments.

Figure 35. TD-IκBα is resistant to TNFα induced degradation and leads to decreased Bcl-2 expression in TNFα treated cells. Jurkat rtTA-Neo, rtTA-2N and rtTA-2NΔ4 cells were incubated with or without doxicycline (Dox 1μg/ml) for 24h to induce expression of the IκBα mutants. Cells were subsequently treated with tumor necrosis factor α (TNFα 20 ng/ml) for 24h. (**A**) Western blot analysis of whole cell extracts was performed using an N-terminal IκBα mAb which identifies both endogenous IκBα and the C terminal truncated 2NΔ4 (lower band) protein. IκBα levels were unchanged by Dox addition to control rtTA-Neo cells (compare lanes 1 and 2) or by TNFα addition for 24h (lane 3). Dox induction of rtTA-2N and rtTA-2NΔ4 cells led to increased IκBα expression (lanes 5 and 8) which was not degraded upon TNFα stimulation (lanes 6 and 9). (**B**) Electrophoretic mobility shift assay (EMSA) analysis illustrated that NF-κB activation by TNFα was blocked in Dox treated cells expressing the TD-IκBα (lanes 8 and 12), but not in control rtTA-Neo cells (lane 4). (**C**) Western blot analysis of Bcl-2 levels also revealed that Dox treated Jurkat rtTA-2N (lanes 7-12) and rtTA-2NΔ4 (lanes 13-18) but not rtTA-Neo (lanes 1-6) cells decreased Bcl-2 levels with increased TNFα incubation.

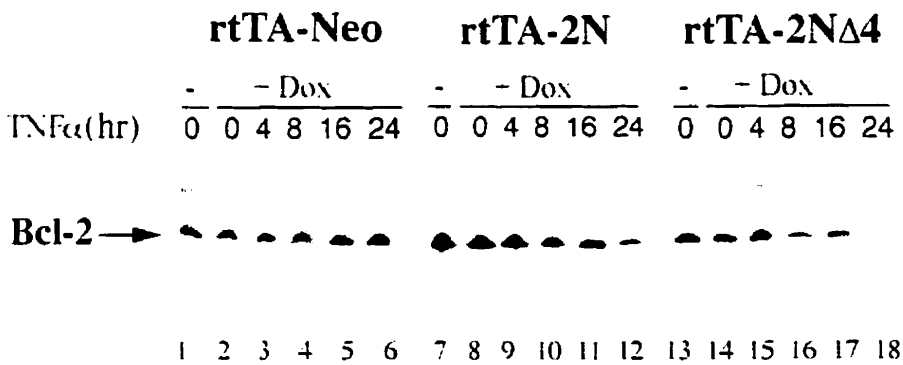
A


Neo
2N
2NΔ4
Dox
- + - + - +
TNFα(hr) 0 0 24 0 0 24 0 0 24
IκBα
2NΔ4
1 2 3 4 5 6 7 8 9

B


rtTA-Neo
rtTA-2N
rtTA-2NΔ4
Dox - - + + - - + + - - + +
TNFα - + - + - + - + - + - +
NFκB
1 2 3 4 5 6 7 8 9 10 11 12

C


rtTA-Neo
rtTA-2N
rtTA-2NΔ4
- - Dox
TNFα(hr) 0 0 4 8 16 24 0 0 4 8 16 24 0 0 4 8 16 24
Bcl-2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

CHAPTER VI

GENERAL DISCUSSION

MECHANISM AND ROLE OF NF-κB ACTIVATION IN HIV-1 INFECTED MYELOID CELLS

1.0 SUMMARY OF RESULTS

Cells of the monocytic lineage are important mediators of HIV-1 disease; they are one of the first cell types infected by HIV-1 during sexual transmission and unlike T cells, resist HIV-induced cell death. They serve as reservoirs of HIV, capable of producing virus throughout their life span and transmitting virus to $CD4^+$ (187) and $CD8^+$ T cells (186). The inherent phagocytic properties of these cells, coupled with their ability to migrate through out the body, likely contributes to the extensive dissemination of virus observed in HIV-1 infection (reviewed in 336). These properties make cells of the monocytic lineage a formidable foe in battling HIV immune destruction. Examination of the signaling pathways perturbed in HIV infected myeloid cells may shed light on the mechanisms by which these cells maintain HIV replication and contribute to the devastation of the immune system caused by HIV.

1.1 Constitutive NF-κB Activation is due to Increased IκBα Protein Turnover Mediated by PKR

Previously, it has been shown that HIV-infected primary monocytes and myeloid cell lines PLB-985 and U937, exhibit constitutive NF–κB activation upon virus infection (112,370,372).We attempted to delineate the mechanism responsible for the constitutive NF-κB activity and found that the regulation of NF-κB/IκBα expression is altered in PLB-IIIB and U9-IIIB. PLB-IIIB cells constitutively express IFNα/β mRNA and exhibit altered kinetics of IFNα/β mRNA induction in response to Sendai virus infection compared to uninfected PLB-985 cells. PKR protein and enzymatic activity were also increased in PLB-IIIB cells compared to uninfected cells as was IκBα protein turnover rates. Moreover, PLB-IIIB cells possessed increased levels of c-Rel and p100/p52 protein compared to PLB-985 and these levels were further augmented by cytokine or phorbol ester stimulation.

Addition of a neutralizing anti-IFNα/β antibody to the culture dramatically reduced the level of PKR expression in PLB-IIIB cells, thus demonstrating a role for low level IFNα/β production in the constitutive activation of PKR. IFNα/β neutralization in PLB-IIIB cells further led to an increase in IκBα protein stability, altering the turnover rate of IκBα to levels seen in uninfected cells. As demonstrated previously, PKR is capable of phosphorylating IκBα *in vitro* and inducing NF-κB binding activity (236,282). The persistent inductive signal provided by PKR may contribute to the constitutive NF-κB binding activity observed in HIV-infected myeloid cells.

Several studies have addressed the mechanism of NF-κB activation in HIV-infected cells. Bachelerie et. al. demonstrated sustained NF-κB binding activity in the chronically HIV-infected promonocytic cell line U937 (25). Increased transcription and processing of the p105 precursor gave rise to increased intracellular pools of NF-κB in these chronically infected U937 cells (24). This activity may be mediated by the HIV encoded protease which cleaves p105 to generate a 45-kDa DNA-binding subunit *in vitro* and in HIV infected T cells (361). Although we also found elevated expression of p105 protein in chronically infected U937 cells, significant increases in p105 protein were not seen in PLB-IIIB cells. These cells, although both myeloid precursor cells, differ in their state of differentiation suggesting that NF-κB1 may be developmentally regulated.

Modulation of intracellular NF-κB protein levels may represent a control mechanism for enhanced activation of NF-κB-regulated gene expression. Enhanced levels of NF-κB2 and c-Rel in PLB-IIIB (and NF-κB2, NF-κB1 and c-Rel in U9-IIIB) cells may result in increased intracellular pools of latent NF-κB. A constitutive inductive signal mediated by PKR would then translate into increased active NF-κB and enhanced κB dependent transcription. The RelA gene is not known to be stimulated in response to mitogenic stimuli including PMA (377) and is not regulated by NF-κB (436). Our results are consistent with

previous observations, since the levels of RelA were only moderately increased in the differentiated PLB-IIIB cells (Fig. 20). Increasing the intracellular levels of distinct NF-κB subunits would permit high levels of HIV LTR directed gene transcription, and could thus provide a favorable environment for HIV replication. Furthermore, changing the relative abundance of NF-κB would also be expected to alter host transcription since several studies have delineated differential transcriptional specificities of NF-κB homo- and heterodimer combinations (reviewed in 373).

The level of IκBα protein was consistently lower in PLB-985 cells compared to PLB-IIIB cells. However, the pool of IκBα in PLB-IIIB exhibited a higher rate of protein turnover (Fig. 14). Increased IκBα protein turnover was also seen in HIV-infected U9-IIIB cells and has been documented in other HIV-infected myeloid cells (25,287), suggesting one mechanism for increased constitutive NF-κB•DNA binding activity in HIV-infected cells (370). Using the proteasome inhibitor MG132, we demonstrated the accumulation of phosphorylated forms of IκBα in both uninfected and HIV-infected cells, although only minor differences in the rates of accumulation were detected (Fig.18). McElhinny et. al. also suggested that the increased turnover compensated for the elevated levels of IκBα protein seen in chronically HIV-infected U937 cells (287). Although our model of chronically HIV-infected U937 cells exhibited lower levels of IκBα protein than uninfected cells, IκBα protein turnover was increased similar to PLB-IIIB cells (Fig. 15). The steady state level of IκBα may thus depend on the relative increase in rate of constitutive IκBα protein turnover versus the increased level of protein synthesis resulting from NF-κB mediated IκBα transcriptional upregulation. Increased IκBα synthesis and turnover, coupled with the elevated levels of NFκB2 p100/p52 and c-Rel proteins, indicates that the NF-κB/IκBα autoregulatory loop is specifically upregulated in HIV-infected myeloid cells.

The observation that IκBα mRNA induction is decreased in PLB-IIIB cells may in part be explained by the differential responsiveness of the IκBα promoter to different NF-κB dimer compositions. The IκBα promoter is transcriptionally activated by the NF-κB subunits RelA and p50, but only to a limited extent by c-Rel (250). While the levels of RelA are not significantly elevated in HIV-infected cells, c-Rel and p100/p52 account for a much greater proportion of the NF-κB pool. Furthermore, activation of IκBα mRNA synthesis peaked earlier in HIV-infected cells compared to non-infected cells (data not shown) and the induction profile was similar to uninfected cells but was shifted to earlier time points. By 6 hours, IκBα mRNA levels were returning to baseline in HIV-infected cells but were peaking in non-infected cells. It therefore appears that NF-κB is more readily activated in HIV-infected cells, possibly as a result of the increased intracellular levels of NF-κB proteins, permitting a more rapid activation of κB regulated genes such as IκBα.

Interestingly, the 30-kDa IκB-like band specific to PLB-985 cells, degraded with kinetics similar to IκBα and exhibited a similar pattern of accumulation of phosphorylated forms in the presence of MG132. In fact, the 30-kDa protein accumulated two distinct phosphorylated species with time after addition of proteasome inhibitor (Fig.18). The phosphoserine 32 IκBα antibody also recognized the 30-kDa species (Fig.27), suggesting that this N-terminal segment responds to stimulus induced phosphorylation. The characteristics of this species are consistent with a degradation product of IκBα, although it could also represent an alternatively spliced form of IκBα or a distinct cross-reactive form. Likewise, it is not known if the 30-kDa protein maintains the capacity for cytoplasmic retention of NF-κB. If the 30-kDa form is a degradation product of IκBα, then the total pool of IκBα in PLB-985 cells would be greater than the pool in PLB-IIIB cells.

Both acute Sendai virus infection and chronic HIV infection lead to the induction of NF-κB DNA-binding activity (371,372), suggesting that the two viruses may converge on a

signaling pathway which leads to NF-κB activation. One common point at which the infection cycle of these viruses may overlap is with the generation of dsRNA during viral replication which may in turn activate the dsRNA dependent kinase (PKR) (294,382). PKR is induced by IFNα/β and is activated by binding to dsRNA molecules generated during viral infection. The antiviral activities of IFN are in part brought about by a PKR-mediated phosphorylation of initiation factor eIF-2α which in turn inhibits protein translation. PKR has been implicated in the activation of NF-κB·DNA-binding activity *in vitro* and *in vivo*. PKR phosphorylated IκBα in a dsRNA dependent manner (322), and activated NF-κB·DNA binding activity in cellular extracts (236). In PKR depleted HeLa cells, NF-κB·DNA binding activity was not activated by dsRNA (poly[I];poly[C]) (282), and PKR deleted mouse embryo fibroblasts exhibited deficient NF-κB signaling upon dsRNA stimulation (237). Increased PKR protein and phosphorylating activity is a likely mechanism by which constitutive activation of NF-κB may be perpetuated in chronically infected cells.

HIV-1 Tat protein was previously shown to downregulate PKR activity in an HIV-infected T cell line and in Tat-expressing HeLa cells (376), although this observation was not consistently observed. Direct physical association between Tat and PKR *in vitro* and *in vivo* in HeLa cells and T lymphocytes has been reported, suggesting a potential mechanism by which HIV could suppress PKR activity specifically, and the interferon system in general (290). In contrast, our results indicate that PKR mRNA, protein and enzymatic levels are upregulated in HIV infected monocytic cells; low level IFNα/β production secreted into the media of HIV-infected cells appears to be sufficient to induce PKR expression. Recent results, obtained in collaboration with the Koromilas group, suggest that kinase deficient PKR, induces NF-κB dependent CAT activity (204). The requirement for an inactive kinase function to induce NF-κB activation may be explained by considering PKR's inhibition of protein translation. Transfection of wild type PKR would

repress CAT translation masking any effect on NF-κB activation. This result also suggests that NF-κB activation may not require the enzymatic function of PKR. One hypothesis is that PKR acts as a scaffolding protein, bringing signaling molecules in close proximity. Such a function for PKR has been reported for signal transducer and activator of transcription (STAT) signaling (461) and NF-κB activation by RIP also does not require kinase function (430), although the kinase domain appears to synergistically activate NF-κB. PKR may function similarly, its kinase domain may not be obligatory for NF-κB activation but may function to augment the level of active NF-κB. We can postulate that PKR's ability to phosphorylate eIF-2α is inhibited in HIV-infected myeloid cells, permitting PKR's activation of NF-κB to be revealed.

Tat is an attractive candidate inhibitor of PKR translational regulation since it has been shown to inhibit PKR phosphorylation of eIF2α *in vitro* (57) while inducing IFNα production (483). Alternatively, cellular inhibitors of PKR may be activated in HIV-infected cells and serve to limit PKR's effect on protein translation. A positive role for PKR in upregulating NF-κB activation, potentially enhancing HIV replication, may explain why elevated blood levels of IFN are present in the absence of visible antiviral effects in HIV-infected individuals (163,368). PKR, normally a mediator of the IFN antiviral response, would function to promote viral replication. PKR may also be more active in HIV-infected cells since HIV TAR RNA activated PKR kinase activity *in vitro* (126,374) and *in vivo* (2). In addition, recent studies have found that TNFα can activate PKR (76,477) via PKR activator (PACT) protein (333); PLB-IIIB and HIV-infected cells in general, secrete low levels of TNFα (104,105,373) which may contribute to the activation of PKR and PKR mediated signaling pathways in these cells. As a result, IκBα protein turnover is augmented in HIV-infected myeloid cells and increased pools of NF-κB subunits may contribute to an environment favoring HIV-1 replication.

1.2 IκBβ Contributes to Constitutive NF-κB Activation: Role for IKK Activation

Since IκBβ has been implicated in persistent NF–κB activation (23,168,210,342,415,427), we sought to characterize its role in maintaining constitutive NF–κB activation in HIV-infected myeloid cells. IκBβ was found complexed to NF–κB in nuclear extracts from HIV-infected cells and in uninfected cells stimulated with various inducers. NF–κB·DNA complexes could not be completely dissociated by GST-IκBα in HIV-infected cells whereas complexes induced by TNFα or PMA were readily dissociated in uninfected cells. Depletion of IκBβ from HIV-infected nuclear extracts resulted in NF-κB·DNA binding complexes that were sensitive to inhibition by GST-IκBα mediated dissociation, indicating that IκBβ protects DNA bound NF-κB from dissociation by IκBα. Furthermore, co-expression experiments demonstrated that IκBβ increased NF-κB dependent gene activity. Finally, IKK was constitutively activated in HIV-infected myeloid cells; interestingly, Sendai virus infection of U937 also leads to prolonged activation of IKK (unpublished results). It is possible that activation of IKK and formation of IκBα resistant IκBβ·NF-κB·DNA ternary complexes is a common mechanism exploited by several viruses to regulate host and viral gene expression.

IκBβ was detected in NF–κB·DNA binding complexes of uninfected U937 and PLB-985 cells after long periods of TNFα or PMA stimulation. In contrast to HIV-infected cells, NF-κB complexes from uninfected cells were sensitive to IκBα mediated dissociation. The reason for this discrepancy may lie in the additional pathways that are activated or inhibited in HIV-infected cells. IκBβ protection of NF-κB may require additional factors (such as HMG) that are activated in HIV-infected myeloid cells. Alternatively, IκBβ mediated protection in uninfected cells may require longer induction periods than those used in this study.

Several studies have implicated IκBβ in maintaining persistent NF-κB activation (23,168,210,342,415,427). B cells stimulated with LPS or IL-1 experienced a persistent degradation of IκBβ that correlated with sustained NF-κB activation, while inducers that did not degrade IκBβ only produced a transient activation of NF-κB (427). A similar correlation between IκBβ degradation and persistent NF-κB activation was also reported in human vascular endothelial cells (210). Although others have observed IκBβ degradation during transient activation of NF-κB (215), persistent activation of NF-κB is generally associated with IκBβ degradation. The increased rate of IκBβ turnover seen in our HIV-infected cells supports a role for IκBβ in maintaining persistent NF-κB activation.

IκBβ degradation has been also implicated in the synergistic activation of NF-κB observed in TNFα and IFNγ stimulated cells (75). Recently, these authors have further shown that PKR mediates the degradation of IκBβ induced by TNFα and IFNγ. Their results also indicate that PKR was involved in activating NF-κB in response to TNFα alone; a transdominant mutant of PKR blocked TNFα activation of an NF-κB reporter plasmid. These results suggest a possible hierarchy of control in cross-talk activation of NF-κB: PKR may be upstream of IKK and regulate sustained NF-κB activation via both IκBβ and IκBα degradation. PKR levels are greatly increased in HIV-infected myeloid cells and by virus infection in general. Infection by HIV-1, Sendai and several other viruses also leads to sustained NF-κB activation and the transcriptional upregulation of specific κB regulated genes necessary for an effective immune response. PKR activation often precedes or coincides with NF-κB activation, placing PKR in a key position to regulate sustained NF-κB activation. Experiments conducted in collaboration with the Koromilas group indicate that mutant PKR expression induces IKK expression (204), supporting a concerted signaling mechanism, whereby activation of PKR induces IKK activity and NF-κB transactivation. Supported by previous work implicating PKR in NF-κB signaling, these results strongly suggest that PKR activates NF-κB via IKK activation. This work does not

however rule out a coincident mechanism whereby PKR directly contributes to NF-κB activation by phosphorylating IκB proteins. Further investigations are necessary to identify the mechanism of PKR activation of NF-κB in HIV-infected cells.

Several groups have demonstrated that hypophosphorylated IκBβ can bind NF-κB·DNA complexes without inhibiting DNA binding (85,288,415,433). Hypophosphorylated IκBβ did not mask the NLS of RelA, permitting NF-κB·IκBβ complexes to enter the nucleus and bind DNA (415). Sites important for regulating the ability of IκBβ to chaperone NF-κB into the nucleus were identified in the C-terminal PEST domain; phosphorylation of Ser313 and Ser315 by CKII prevented IκBβ from associating with NF-κB·DNA complexes (288), and conversely, mutation of these sites to alanines permitted IκBβ to form ternary complexes with NF-κB and DNA. Other serines in the PEST domain also appear to be important, since replacing Ser313 and Ser315 with phosphomimetics was not sufficient to block ternary complex formation (288). The IκBβ CKII mutant (S313/315A) also blocked the capacity of IκBα to dissociate NF-κB from DNA (288). Based on these results, it seems likely that the IκBβ complexed with nuclear NF-κB in HIV-infected cells is hypophosphorylated.

In accord with studies conducted by Hirano and colleagues (191), two isoforms of IκBβ of 43- and 41-kDa were detected. The 41-kDa isoform of IκBβ resisted degradation by several inducers in both infected and uninfected cells, while the constitutive protein turnover of the 43-kDa form was increased in HIV-infected cells. One plausible explanation is that virus infection represents the persistent activation signal required for the continuous degradation of IκBβ. Several HIV-1 proteins are known to activate NF-κB; Nef induces NF-κB activation when expressed at the cell surface (41) and gp120 can signal NF-κB by engaging the CD4 receptor in a pathway that involves $p56^{lck}$ (58). Tat is also known to activate NF-κB and leads to TNFα synthesis via NF-κB transcriptional activation (114). Inductive

signals from one or a combination of these proteins may account for the NF-κB activation seen in HIV-infected myeloid cells. Similarly, LPS induced a prolonged NF-κB activation in 7OZ/3 pre B cells as a result of a persistent activating signal that could be blocked by employing antioxidants (427). Constitutive activation of NF-κB may also result due to decreased cellular antioxidant levels (209); HIV-1 infection can lead to decreases in antioxidant levels (404). Increased turnover due to constitutive stimulation could also explain the decreased steady state level of the inducer sensitive 43-kDa IκBβ isoform in HIV-infected cells. Other mechanisms likely exist to maintain IκBβ in a hypophosphorylated form, since antioxidant treatment or proteasome inhibition did not affect the nuclear localization of hypophosphorylated IκBβ in WEHI 231 cells (342). Maintenance of hypophosphorylated IκBβ in these cells may result from the activation of a phosphatase, since treatment with the phosphatase inhibitor okadaic acid led to IκBβ hyperphosphorylation.

IκBβ protection of DNA bound NF-κB does not appear to involve specific NF-κB subunits since the composition of DNA bound NF-κB is similar before and after immunoprecipitation (Fig. 24C). This does not, however imply that all genes are protected equally. IκBβ may differentially protect DNA bound NFκB from IκBα mediated dissociation, thus protecting only a subset of genes and providing an additional level of regulation. This conjecture is supported by the observation that only a portion of the constitutive NF-κB DNA binding activity is lost by IκBβ immunodepletion (Fig. 24A and B).

IκBβ protection of DNA bound NF-κB may also explain why PLB-IIIB cells have higher levels of IκBα protein than uninfected PLB-985 cells yet manage to exhibit constitutive NF-κB activation. Although these cells exhibit increased IκBα protein turnover, IκBβ

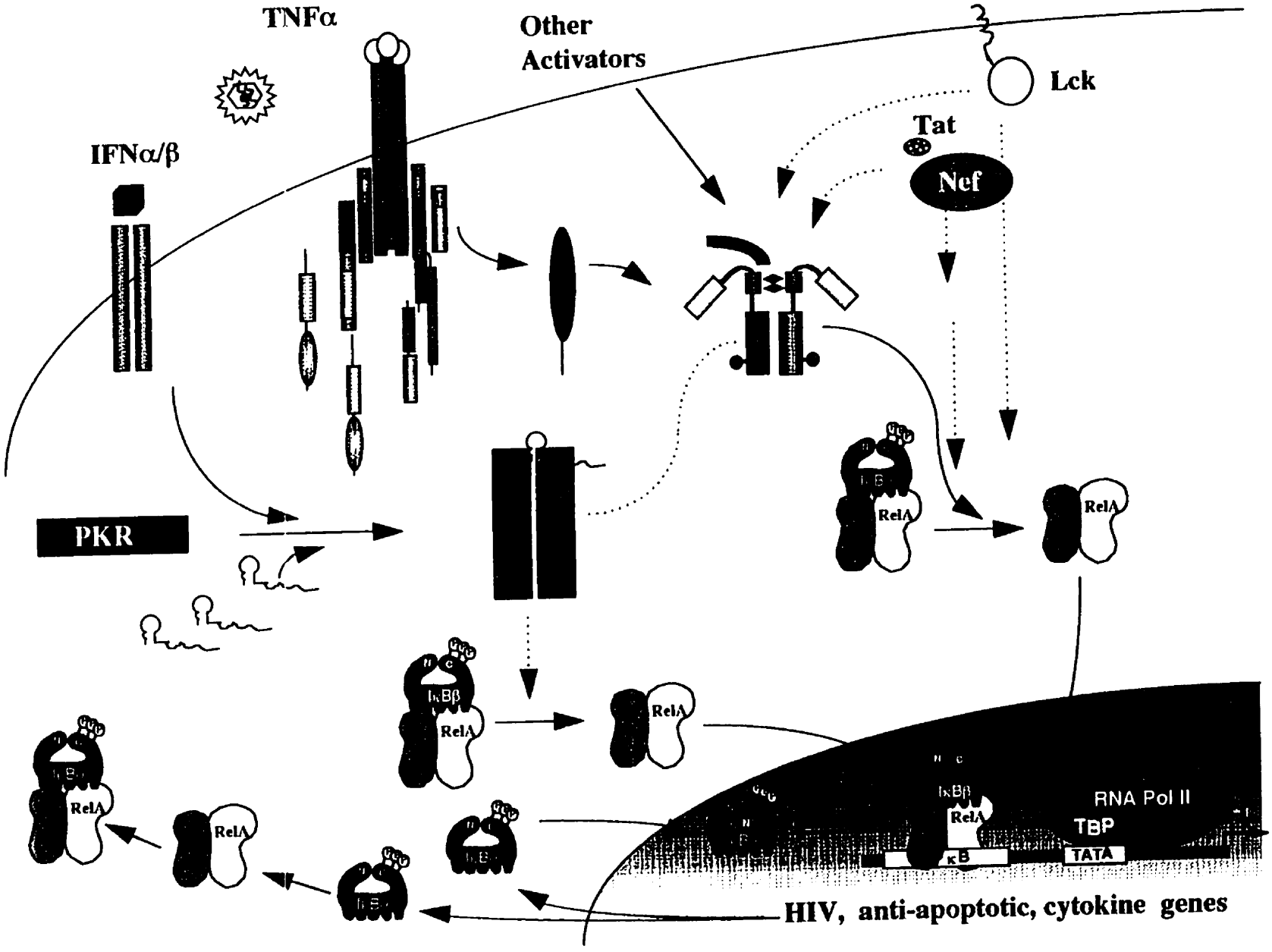
shielded nuclear NF-κB may provide PLB-IIIB cells with an additional mechanism to maintain high levels of constitutive NF-κB activation.

We can then speculate that the induction of PKR and subsequently IKK, arising as a consequence of the low level cytokine (104,105,373) production, HIV-1 inductive signals or due to the increased pro-oxidant state of HIV-infected cells (404), leads to increased phosphorylation and degradation of IκBα and IκBβ. Both proteins release active NF–κB which translocates to the nucleus and transcriptionally activates responsive genes. Newly synthesized IκBβ, in addition, enters the nucleus and prevents IκBα mediated termination of the NF–κB response. Specific κB dependent genes may be maintained in a transcriptionally active state. This could create an environment conducive to viral replication, promoting HIV-1 LTR driven gene transcription. Figure 36 illustrates this model of NF-κB activation in HIV-infected cells.

1.3 NF-κB Protects Cells from HIV-Induced Apoptosis

NF-κB has also been implicated in the regulation of stimulus induced apoptosis, inducing cell death in some circumstances (9,29,213,262), and protecting from cell death in others (47,437,444). We therefore investigated whether the constitutive NF–κB activation, seen in HIV-infected myeloid cells as a result of the altered regulation of NF–κB/IκB proteins, is involved in apoptotic signaling. Our results indicate that TNFα induces apoptosis in U937/U9-IIIB and PLB-985/PLB-IIIB cells and the extent of cytotoxicity is inversely correlated with the level of NF-κB activation. Inhibition of TNFα induced NF-κB activation using the anti-oxidant N-acetyl cysteine (NAC) increased apoptosis in both infected and non-infected cells. Conversely, deliberate activation of NF-κB by the non-cytotoxic inducer IL-1β, partially rescued cells from TNFα/CHX induced apoptosis. In

Figure 36. Proposed mechanism for NF-κB activation in HIV-1 infected myeloid cells. PKR is induced by low level IFN and possibly TNFα production. IKK maybe downstream of PKR, activated directly by HIV-1 proteins or activated indirectly by secreted TNFα. These kinases lead to the phosphorylation and degradation of IκBα and IκBβ, releasing NF-κB to translocate to the nucleus and transactivate responsive genes. Newly synthesized IκBβ enters the nucleus and prevents IκBα mediated termination of the NF-κB response maintaining NF-κB mediated transcriptional activation of select genes. This could create an environment conducive to viral replication, promoting HIV-1 LTR driven gene expression and Rev mediated mRNA transport.


TNFα
Other
Activators
Lck
Tat
Nef
IFNα/β
PKR
RelA
IκBβ
RNA Pol II
TBP
TATA
κB
HIV, anti-apoptotic, cytokine genes

T cells, NF-κB antagonized TNFα induced apoptosis and conversely its inhibition exacerbated TNFα cytotoxicity.

Interestingly, NAC incubation alone or CHX/NAC induced HIV-infected cells to undergo apoptosis. HIV-infected monocytic cells may receive a persistent apoptotic signal that is countered by the maintenance of a redox sensitive, NF-κB induced, anti-apoptotic protein. Although HIV-infected cells constitutively express NF-κB, they are still more sensitive to TNFα mediated cytotoxicity. This may suggest that HIV-1 infection propels an apoptotic pathway in myeloid lineage cells that is countered by NF-κB activation. Lymphocytes from HIV-infected individuals have a high level of background apoptosis upon *ex vivo* culture and initiate apoptotic signaling in response to activation by polyclonal inducers (170). Indeed, several HIV-1 proteins have been shown to increase the sensitivity of HIV-infected cells to apoptotic stimuli. Env gp160/gp120 has long been known to induce apoptosis in T cells (248,277). Tat induces apoptosis and increases sensitivity to apoptotic signals by upregulating caspase 8 (40); Nef sensitizes $CD4^+$ T cells to apoptosis via upregulation of Fas/FasL pathway; and Vpu expression increases the susceptibility of T cells to Fas killing (67). Similar pathways may be involved in HIV-infected myeloid cells. The resetting of an apoptotic threshold may hence make cells more sensitive to further cytotoxic insults since an apoptotic pathway is ready to be executed.

The effect of NAC on chronically infected U9-IIIB cells may be similar to its effect on TNFα induced apoptosis. Productive HIV-1 infection leads to increased TNFα expression which may stimulate cells by an autocrine regulatory mechanism. Constitutive expression of TNFα mRNA is present in U9-IIIB but not U937 cells and can be induced to higher levels by mitogen stimulation in HIV-infected cells (241). Also U9-IIIB cells strongly upregulate IL-1β gene transcription and protein production in response to PMA stimulation, while U937 cells do not express detectable IL-1β mRNA (105). Increased

TNFα and/or IL-1β levels would activate NF-κB and protect cells from HIV-1 induced apoptosis. Alternatively, HIV-1 infection may affect another apoptotic pathway that is sensitive to NF-κB inhibition.

Activation of NF-κB by various stimuli including TNFα, which transduce their activation signal through radical oxygen intermediates (ROI), can be inhibited by pretreating cells with the antioxidant NAC. NAC is thought to inhibit signaling directly through its capacity as a ROI scavenger as well as through its ability to replenish cellular glutathione (GSH) levels (405). Replenishing GSH strongly suppressed HIV-1 replication in human macrophages (159) as did NAC addition in latently infected cell lines (364) and PBMC (365). Others have found that NAC did not affect HIV-1 replication in chronically infected promonocytic U937 cells (11) or actually stimulated HIV-1 virus replication in monocyte derived macrophages (320). The discrepancy in these findings may result from differences in concentration of NAC or differences in experimental procedures.

Murine macrophages and fibroblasts deficient in the prototypical transactivating NF-κB subunit RelA, are highly sensitive to TNFα induced apoptosis (465), and reintroduction of RelA repressed the toxic effects of TNFα. Membrane interaction with antibodies against IgM in WEHI 231 B cells also induces apoptosis that is exacerbated by the inhibition of NF-κB (465). Ectopic expression of c-Rel rescued these cells from anti-IgM induced apoptosis while microinjection with a GST-IκBα fusion protein or anti c-Rel antibody induced cell death. Interestingly, c-Rel protein levels were dramatically increased in both U937 and U9-IIIB cells upon TNFα stimulation. It will be interesting to determine if c-Rel is involved in protecting these cells from apoptosis.

The anti-apoptotic effects of NF-κB are supported by experiments in Jurkat cells which are engineered to inducibly express transdominant IκBα repressors (TD-IκB) (239). Normally

resistant to TNFα toxicity, Jurkat cells treated with TNFα underwent rapid apoptosis when NF-κB induction was abolished by the induction of TD-IκB (Fig. 34). Similar results were obtained by Verma and colleagues (437) who examined TNFα induced apoptosis in cells constitutively overexpressing a transdominant IκBα mutant harboring the Ser-32/36-Ala modification in the signal response domain. Both the sensitivity and the kinetics of TNFα induced apoptosis were enhanced in the cell lines tested (437). Together with the previous studies, our results indicate that S32/36A mutations are sufficient to confer sensitivity to TNFα induced apoptosis. Additionally, Jurkat cells pretreated with NAC, in the absence of TD-IκB expression, exhibited heightened sensitivity to TNFα/CHX induced apoptosis (Table 4). These results suggest that the effect of NAC on TNFα induced apoptosis in HIV-infected and non-infected U937 cells is due to inhibition of NF-κB activation.

Various NF-κB regulated anti-apoptotic gene products are known to protect cells from programmed cell death, including the zinc finger protein A20 (206,424), p53 (464), IAP proteins (86) and members of the Bcl-2 family (reviewed in 356,470). Expression of A20 in Jurkat cells expressing a transdominant IκBα mutant did not restore protection from TNFα induced apoptosis (437), suggesting that it is either not the target of NF-κB transactivation or that it acts in cooperation with other anti-apoptotic genes.

Bcl-2 expression counters apoptotic signaling by a vast array of inducers in many cell models (reviewed in 470). Several Bcl-2 proteins have been identified in mammals including Bcl-x_L, Bcl-w and Mcl-1 which inhibit apoptosis and Bax, Bik, Bak, Bad and Bcl-x_S which activate apoptosis. Cell fate is determined by the relative ratio of anti- and pro-apoptotic members. Decreased Bcl-2 levels have been reported to account for the induction of apoptosis by HIV-1 infection in some cell systems (116,410). Although Bcl-2 levels were comparable in infected and non-infected cells, the level of Bax was elevated in HIV-infected U937 cells, suggesting that increased Bax expression may contribute to the

rapid induction of apoptosis observed in HIV-infected cells. Bcl-2 expression decreased in TNFα treated Jurkat cells which were deficient in NF-κB induction because of TD-IκB expression but not in cells in which NF-κB could be activated. Bcl-2 levels were also modulated by TNFα and NAC treatments in U937 and U9-IIIB although the decreases were not dramatic. This was particularly true in non-infected cells suggesting that other NF-κB regulated proteins are likely important in protecting these cells from TNFα induced apoptosis.

The apoptosis inhibitor proteins, c-IAP1 and c-IAP2, and the TRAF family members, TRAF-1 and TRAF-2, have recently been identified as the targets of NF-κB anti-apoptotic signaling pathways involved in the suppression of TNFα induced cell death (86,445). The c-IAP proteins directly inhibit caspase activation, blocking death signaling pathways. The c-IAP proteins and TRAF-2 also activate NF-κB establishing an autoregulatory loop, maintaining their transcriptional activation. It will be interesting to determine if c-IAP and TRAF protein profiles are deregulated in HIV-infected myeloid cells.

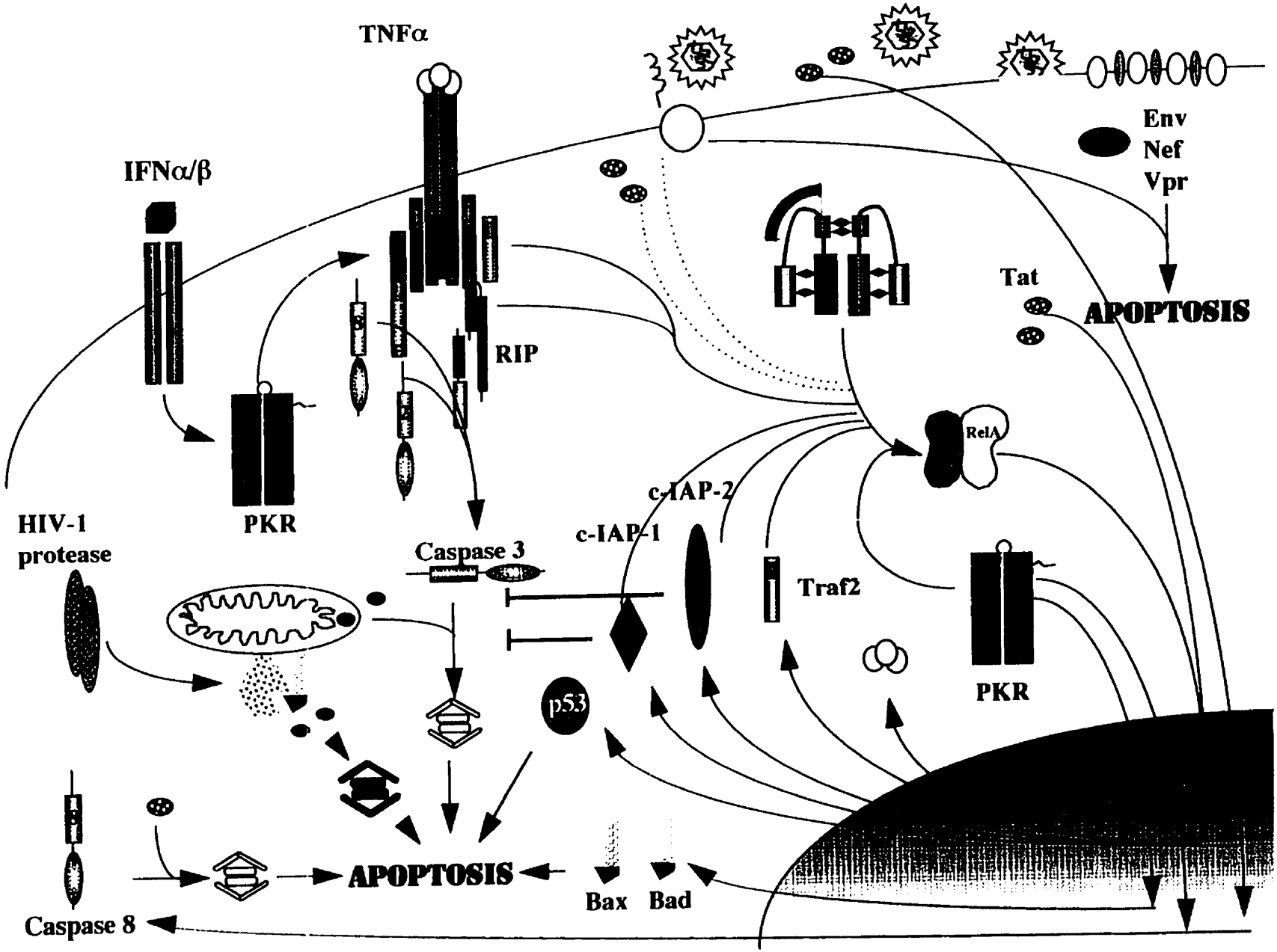
Studies have also found roles for p53 and PKR in TNFα induced cell death. Activation of PKR upregulates p53 expression in U937 and increases the sensitivity of these cells to TNFα activated apoptosis (477). Correspondingly, expression of transdominant mutant PKR interferes with TNFα apoptotic signaling. PKR activation is tightly regulated and precedes p53 upregulation. The authors suggest that PKR may activate p53 via NF-κB; the p53 promoter contains a κB site and NF-κB activates p53 (464). Although NF-κB is involved in activating anti-apoptotic genes, TNFα often results in cell death in the face of sustained NF-κB activation. It is conceivable that NF-κB activates protective genes until a threshold level of p53 (or other apoptotic inducer) is activated, NF-κB regulated anti-apoptotic proteins are no longer able to suppress TNFα induced death pathways and the cell is propelled into apoptosis.

Other studies have found that dsRNA activation of tetracycline inducible PKR leads to increased Fas, TNFR1, FADD, Flice, Bad and Bax expression while catalytic mutants of PKR abrogate induction of these proteins (34). The induction of apoptosis did not depend on PKRs ability to inhibit protein translation since dsRNA stimulation in the presence of chemical protein translation inhibitors did not result in the same phenotype. PKR therefore appears to be involved in activating NF-κB and propagating TNFα induced apoptosis. Conceivably PKR activation of NF-κB could activate genes such as Fas, TNFR1 and FADD, as well as genes involved in preventing apoptosis. The conflicting agendas would battle until a threshold was reached and the cell's fate decided by the stronger of the signals.

2.0 MODEL FOR NF-κB ACTIVATION IN HIV-INFECTED CELLS

Based on these results, we can propose a model (Fig. 37), in which HIV-1 infection leads to the production of IFNα/β, TNFα and dsRNA (i.e. TAR) and the subsequent activation of PKR. PKR, HIV-1 proteins or pathways activated by HIV-1 induce NF-κB signaling pathways at least some of which involve IKK, leading to increased IκBα and IκBβ degradation and nuclear NF-κB. NF-κB activates the transcription of TNFα, which may introduce a feed-back loop, propagating NF-κB activation, as well as the activation of numerous other genes including anti-apoptotic genes and p53. PKR further contributes by activating pro-apoptotic Bcl-2 family members and components of the Fas death pathway. Other HIV-1 proteins such as Tat (40), gp160/120 (37) and Nef (485) may also contribute by inducing NF-κB activation as well as apoptotic pathways. These apoptotic players reset the apoptotic threshold of the cell, so that sustained NF-κB activation is required to maintain cell viability. This may be more attainable for cells of the myeloid lineage, since these cells are typically involved in mediating cell death of infected or otherwise doomed

Figure 37. Model for functional hypothesis of NF-κB activation in HIV-1 infected myeloid cells. NF-κB is activated to counter the numerous apoptotic signals activated in HIV-1 infected cells. PKR leads to NF-κB activation as well as activation of several pro-apoptotic Bcl-2 proteins and components of the Fas death signaling pathway. HIV-1 proteins Tat, Nef and Vpu may additionally propel cells to undergo apoptosis. These proteins may reset the cells apoptotic threshold, making them more likely to commit suicide. NF-κB is activated possibly in response to secreted TNFα or through signaling pathways activated by HIV-1 proteins, culminating with IKK activation. NF-κB counters the apoptotic pathways by activating genes that promote cell survival, maintaining cell viability in spite of active death signaling pathways.


TNFα
IFNα/β
Env
Nef
Vpr
Tat
APOPTOSIS
RIP
RelA
c-IAP-2
c-IAP-1
HIV-1
protease
PKR
Caspase 3
Traf2
PKR
p53
APOPTOSIS
Bax
Bad
Caspase 8

target cells, and must hence be more resistant to the apoptotic inducers they secrete.Monocytic resistance to apoptotic induction may be the basis for the differential effect of HIV-1 infection on T cell and monocyte cell death.

3.0 FUTURE DIRECTIONS

The experiments presented in this thesis examine the regulation and function of the NF-κB/IκB system in two chronically HIV-infected monocytic cell models. The results suggest that NF-κB activation is perturbed due to the activation of upstream kinases which result in enhanced degradation of IκBα and IκBβ, and the activation of NF-κB. These cells resist HIV-induced apoptosis and require constitutive NF-κB activity to maintain cell viability. The results obtained in PLB-IIIB and U9-IIIB cells must be verified in primary cell models of HIV-1 infection. Although studies have shown that HIV-infected macrophages express constitutive NF-κB activation (25,313,416), the mechanism of this activation has not been established. Similar experiments to the ones described could be performed on monocytes from healthy, asymptomatic and symptomatic HIV-infected individuals to assess whether PKR and IKK are constitutively active. It would also be interesting to determine if nuclear extracts from HIV-infected monocytic cells contain IκBβ·NF-κB·DNA ternary complexes.

Several questions remain regarding the model of NF-κB activation proposed in this thesis. It would be interesting to determine if PKR can directly activate IKK. If yes, does PKR require, IFNα/β and dsRNA, TNFα or Tat to be activated? Some evidence suggests that PKR may directly phosphorylate IκBα (236) or IκBβ (76); although intriguing, this observation requires further study.

Our experiments indicating a role for NF-κB in protecting HIV-infected monocytic cells from apoptosis, should also be confirmed in primary cell models. The consequences of

inhibiting NF-κB activation in macrophages exposed to HIV-1 has been examined although these studies have reported conflicting results. Early experiments found that NAC inhibited RT activity in primary cord blood and adult donor monocyte-derived macrophages exposed to M-tropic virus and that this effect persisted for up to 35 days after exposure to NAC (196,267). Other studies have contradicted these findings (70,320) and hence more rigorous investigations are necessary. Inhibitors of NF-κB which exhibit greater specificity, may help to resolve this question.

Although our experiments indicate HIV-infected monocytic cells are more sensitive to apoptotic stimuli and that modulation of Bcl-2 family proteins may be involved, our experiments do not exhaustively examine the veritable cornucopia of apoptotic signaling molecules that may be involved. It would be interesting to investigate if upstream caspases are activated, poised and ready to amplify succeeding signals. Several examples support such a putative mechanism. Tat induces apoptosis and increases sensitivity of T cells to apoptotic signals by upregulating caspase 8 (40), ICE is activated in CD4+ lymphocytes from HIV-infected individuals (400) and HIV-infected T cells express increased levels of Fas (397) and FasL (300). Similar mechanims may be activated in HIV-infected myeloid cells.

Several NF-κB subunits have been identified as important mediators of the NF-κB anti-apoptosis response to different cell death inducers; RelA is important in preventing apoptosis in liver cells during development (465) and c-Rel can prevent anti-IgM induced apoptosis in B cells (465). c-Rel was highly expressed in our HIV-infected cell models and its expression was dramatically increased by mitogenic stimulation. c-Rel may thus be a critical component of the NF-κB response to protecting cells from apoptosis in HIV-infected myeloid cells.

IκBβ may differentially protect DNA bound NF-κB depending on the promoter context or other, yet undefined factors. IκBβ may specifically protect κB dependent genes involved in mediating the NF-κB anti-apoptosis response. Since continual degradation and resynthesis of IκBβ is necessary for generating hypophosphorylated IκBβ, the form likely involved in sustaining NF-κB·DNA binding, HIV-infected cells treated with NF-κB or protein synthesis inhibitors may activate apoptosis as a result of diminished hypophosphorylated IκBβ generation. According to this hypothesis, HIV-infected cells which are exposed to a persistent apoptotic signal, would exhibit sensitivity to agents that inhibit IκBβ degradation (such as NAC) or chemicals that prevent IκBβ synthesis (such as cycloheximide). Uninfected cells which are not receiving an apoptotic signal, would not undergo apoptosis when incubated with such agents. It would be informative to investigate which factors or contexts, if any, are required for IκBβ sustained NF-κB activation.

The genes activated by NF-κB which prevent cell death also remain to be identified. Candidate molecules identified in other models may also effect NF-κB apoptotic protection in HIV-infected monocytic cell lines. Identifying these molecules may identify targets for therapeutic intervention; eradicating long lived HIV-producing cells is a goal of present therapeutic interventions.

Furthermore, it would be interesting to elucidate whether other viruses similarly usurp the NF-κB pathway for maintaining cell viability. Prevention of apoptosis by constitutive activation of NF-κB has been reported in a human cutaneous T cell lymphoma cell line (164); cutaneous T cell lymphomas are rare lymphoproliferative diseases, which are frequently suspected to be caused by viruses (150). Similar to our results, inhibition of constitutive NF-κB induced apoptosis and rendered cells sensitive to TNFα induced apoptosis. HTLV-1 transformed cells also exhibit constitutive NF-κB activation (164), although the effects of NF-κB inhibition of apoptotic signaling is unknown. Sendai virus

infection of myeloid also shares several similarities with HIV; Sendai virus activates PKR in uninfected PLB-985 cells (Fig. 12) and induces IKK activation in U937 cells (unpublished result). It seems plausible that NF-κB mediated protection from apoptosis is a mechanism exploited by different or a subset of viruses (i.e. RNA viruses).

4.0 IMPLICATIONS OF MONOCYTE RESISTANCE TO APOPTOSIS IN HIV-1 PATHOGENESIS

Macrophages and cells of the monocyte lineage are infectable by HIV and are important perpetrators of viral spread. Our results suggest that perturbation of the NF-κB signaling pathway permits monocytic cells to resist HIV-1 induced apoptosis. This resistance contributes to their ability to spread disease particularly during coinfection with opportunistic infections where macrophage production of HIV-1 is dramatically increased. Orenstien and colleagues found that monocytic cells are activated and recruited to the lymph node upon pathogen costimulation where they produce vast quantities of virus that can infect susceptible cells. This observation has two important implications: monocytic cells are indeed important perpetrators of viral spread and that large pools of HIV-infected myeloid cells exist in the host, waiting to be activated.

HIV-1(+) monocytes have also been identified as a source of cellular HIV virus in peripheral blood. Analysis of PBMC from HIV seropositive and seronegative individuals receiving various regimens of antiretroviral therapy, using a novel fluorescence *in situ* hybridization technique, found that the majority of HIV-positive patients harbored monocytes that were positive for HIV RNA. Furthermore, a significantly higher proportion of individuals with higher plasma viral load carried positive monocytes compared to patients with lower viral load (334). A second study found that levels of soluble CD14, a marker for monocyte/macrophage activation, were elevated in all clinical

stages of HIV-1 infection with highest levels present in individuals with AIDS (260). The increased levels of soluble CD14 also significantly correlated with the degree of immunodeficiency and level of HIV-1 replication. The higher viral load may be a direct consequence of virus produced by monocytic cells or due to activating agents secreted by these cells which act on adjacent cell populations. These results suggest that monocytes are a major source of cellular HIV virus even in the absence of pathogen costimulation and infection of monocytic cells may correlate with disease progression.

Monocytic cells may themselves be persistently activated as a direct result of HIV infection. Several lines of evidence suggest Tat to be an important activator of myeloid cells. Secreted Tat has been shown to specifically activate monocytic cells through the upregulation of TNFα and IL-1β (243), and this mechanism involves Tat mediated activation of NF-κB (114). Tat also upregulates CCR5 and CXCR4 chemokine expression in PBMC; CXCR4 was induced in both lymphocytes and monocytes while CCR5 expression was induced specifically in monocytic cells (201). Tat also has β-chemokine-like function and can interact with, and activate, chemokine receptors (12). Tat strongly recruits monocytes and macrophages to sites of active HIV replication, and again, this function likely involves NF-κB activation (158,475). HIV Env proteins may also contribute to monocyte/macrophage activation; recombinant gp120 is known to stimulate monocytic cells (260). Hence, similar to our *in vitro* observations using chronically infected cells, HIV directly activates monocytic cells, potenially leading to NF-κB activation and enhanced viral replication. HIV production by monocytic cells would therefore contribute to *de novo* infection of target cells in the periphery as well as the induction of apoptosis of peripheral blood uninfected $CD4^+$ and $CD8^+$ T cells (see below).

Evidence suggests that HIV-1 induced $CD4^+$ (187) and $CD8^+$ (186) T cell apoptosis depends on monocytic cells. Chronically infected monocyte derived macrophages induce

apoptosis when cocultured with T cells through a mechanism that does not involve viral transmission (165). Other studies have found that macrophages significantly increase the level of bystander apoptosis in uninfected T cells during HIV-1 infection of mixed T cell populations (187). These observations directly implicate monocytic cells in T cell immune destruction and highlight the importance of this cell type in HIV-1 mediated cell destruction.

HIV-infected and immune stimulated monocytic cells are also implicated in mediating neuronal apoptosis causing AIDS dementia. Peripheral blood macrophage/monocytes enter the brain, leading to infection of microglia, the resident macrophage of the brain. They secrete excitants and neurotoxins which induce neuronal injury and apoptotic cell death (103,319,338). The monocytic cells are protected from the apoptotic inducing agents they produce, while neuronal cells succumb to these deadly secretions.

Host factors affecting monocytic cell function may also contribute greatly to HIV pathogenesis. Increased levels of monocyte/macrophage activating factors may spur disease progression while less activated environments may result in reduced T cell depletion and viral spread, giving the immune system time to mount a more effective anti-viral response. Persistent TNF activation during HAART has been associated with treatment failure (24) and one study found that the TNF c2 microsatellite allele strongly associated with the rate of disease progression (224). Activation of the TNF system may permit persistent activation of monocytic cells and continued replication of virus in sites not easily controlled by HAART protocols, such as the brain. Evidence for this stems from the observation that some individuals develop dementia in spite of adhering to HAART regimens and others develop subtle neurobehavioural conditions that impact the quality of their prolonged lives (443).

Additionally, the observation that individuals lacking functional CCR5 expression are almost completely resistant to HIV infection, suggests that monocytic cells play a pivotal role in the early stages of infection. Experiments conducted in mice have revealed that HIV-infected monocytes can travel through the vaginal epithelium, through the underlying connective tissue and into the lymphatic system. Their resistance to HIV-induced apoptosis allows the dissemination of virus from the source of entry to distant sites around the body. Together, these results suggest that myeloid cells are important mediators of HIV-1 pathogenesis, encouraging viral propagation and disease progression throughout the course of HIV-1 infection. Myeloid cells may be particularly important in late stage disease where they account for a significantly greater proportion of infected cells due to the depletion of T cells. This may explain why opportunistic pathogens are particularly deadly to HIV-infected individuals: their depressed immune system fails to contain the infection and stimulation of macrophages results in uncontrolled HIV production, damaging any remaining T cell function. Investigating therapeutic regimens which additionally target myeloid cells may identify more efficacious approaches to obstructing HIV-1 immune destruction which may be crucial to limiting viral dissemination and battling the devastation of AIDS.

4.1 Therapeutic Interventions to HIV: Targeting Cells of the Monocytic Lineage.

Triple combination therapies have provided significant clinical benefit to individuals infected with HIV-1. The failure of these regimens to indefinitely suppress HIV replication suggests that either a low level of infection persists or that latently infected cells remain throughout treatment. The vast majority of clinical trials have measured $CD4^+$ T cell numbers and serum HIV-1 RNA levels. For the most part, they have not investigated the effect of treatments on cells of the monocytic lineage. Recent evidence suggests that a

residual pool of HIV-infected cells are not affected by classical HIV treatments. A preliminary study found that IL-2 and IFN-γ treatment of individuals with HIV-1 RNA concentrations of less than 20 copies/ml, taking five drug therapy, activated replication of latent virus (242). The drug regimen subsequently cleared the activated virus and resulted in a decrease in proviral DNA levels in peripheral blood lymphocytes and lymph nodes. The authors suggest that a subset of both HIV-1-infected lymphocytes and macrophages persist during HAART which must be targeted for HIV-1 eradication. A second study found that HIV-1 was detectable in macrophages even after one year of HAART treatment and was detectable in individuals with virus levels below detection in lymphocytes and in plasma (481). Long lived cells of the monocytic lineage that are readily activated by a number of situations may explain why these therapies fail. The critical function played by cells of the monocytic lineage justify examining the efficacy of therapeutic interventions on this population and suggest that treatments should be tailored to combat monocyte/macrophage viral replication .

Methods to target antiviral nucleotide analogues to macrophages have been developed. Targeting monocytic cells requires that particular features be considered. Macrophage phosphorylation of several antiviral nucleoside analogues, which require phosphorylation for activity, is reduced compared to other peripheral blood mononuclear cells (337,360). To circumvent this problem, Magnani and colleagues developed a drug targeting system that allows the direct administration of nucleotide inhibitors in the phosphorylated form. They engineered red blood cells to carry phosphorylated nucleotide analogues and selectively modified the erythrocyte membranes to permit macrophage recognition and phagocytosis. Red blood cells carrying phosphorylated drug reduced macrophage production of HIV-1 to almost undetectable levels (275) and *in vivo* administration of drug loaded erythrocytes reduced infectivity and disease progression in mice infected with a murine immunodeficiency virus. Used in combination with antiviral drugs that inhibit HIV-

1 replication in lymphocytes, this drug targeting system has the potential to dramatically affect HIV-1 induced disease.

Other strategies have looked at blocking or decreasing expression of the HIV co-receptor, CCR5. Treatment of monocyte derived macrophages with cAMP elevating agents, result in a loss of CCR5 expression, decreased chemotaxis and resistance to HIV-1 infection (426). IL-2 also inhibits HIV-1 replication in human macrophages by downregulating expression of CD4 and CCR5 (238). This strategy has the added benefit of restoring immune function and number of $CD4^+$ T cells. Therapeutic strategies involving CCR5 are attractive for several reasons. Individuals homozygous for a deletion which precludes CCR5 surface expression are not only protected from M-tropic HIV-1 infection but exhibit no adverse phenotype as a result of the deletion.

Our results suggest that blocking NF-κB activation in HIV-infected monocytic cells would induce apoptosis. Although seemingly attractive, global inhibition of NF-κB activation would likely have detrimental effects on the host since many immunological response pathways require NF-κB activation. Specific inhibition of NF-κB activation in HIV-infected monocytic cells could however aid attempts at viral eradication. Experiments that have focused on inhibiting HIV-induced apoptosis suggest that cell death may serve as a host mechanism to limit HIV-1 replication. PBMC exposed to HIV-1 in the presence of caspase inhibitors exhibited increased viral replication and treatment of activated PBMC from asymptomatic HIV-infected individuals with caspase inhibitors stimulated endogenous virus production (79). Several potent chemical inhibitors of NF-κB exist, including NAC, MG132 and salycilate acid (42). Specifically targeting HIV-infected cells could prove challenging. Although the above described method using red blood cells to target macrophages is a potential delivery system, all macrophages would be susceptible to NF-κB inhibition. A retroviral system utilizing specific chemokine receptors could

transport a transdominant IκB molecule which would effectively limit NF-κB activation as shown by our studies using Jurkat IκB mutant expressing cell lines (Fig. 35). Retroviral vectors containing CD4 and HIV chemokine co-receptors have been developed which target HIV- and SIV- infected cell lines and monocyte derived macrophages with similar specificity to the HIV envelope glycoprotein (133). These vectors provide exciting opportunities to deliver antiviral genes directly to HIV-infected cells and enhance current antiviral therapies.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

Analysis of two chronically HIV-infected myeloid cell models and their parental cell lines, have permitted the molecular dissection of NF-κB/IκB regulation in HIV-infected monocytic cells. Using the U937 promonocytic and PLB-985 myelomonoblastic cell lines and their HIV-infected counterparts, we investigated the contribution of altered IκBα and IκBβ regulation to the constitutive NF-κB activity seen in these HIV-infected cells. We further examined the activation of upstream kinases and identified a role for constitutive NF-κB in maintaining cell viability of HIV-infected cells.

1) HIV-infected cells exhibit altered signaling of NF-κB. PKR is increased in HIV-infected cells and this increase results from constitutive low level IFNα/β production. The IκB kinase (IKK) is also constitutively active and together, the pathways affected by these kinases lead to constitutive NF κB activation.

2) The expression of several NF-κB subunits is elevated in HIV-infected cells. PLB-IIIB cells possess increased levels of c-Rel and p100/p52 protein compared to PLB-985 and these levels are further augmented by cytokine or phorbol ester stimulation. U9-IIIB cells additionally express increased levels of p105. Increased amounts of latent NF-κB may contribute to upregulation of the NF-κB signaling pathway in HIV-infected cells.

3) IκBα protein turnover is elevated in HIV-infected myeloid cell lines and this increased degradation correlates with increased PKR expression. Constitutive and inducer mediated degradation of IκBβ protein is also increased in HIV-infected cells. Enhanced IκB degradation is one mechanism by which HIV infection induces constitutive NF-κB in HIV-infected cells.

4) IκBβ is found complexed to NF-κB in HIV-infected nuclear extracts and this activity can be induced upon prolonged TNFα or PMA stimulation in uninfected cells. IκBβ physically protects NF-κB·DNA binding from inhibition by IκBα and protected NF-κB is transcriptionally active. IκBβ protection of NF-κB is an additional mechanism by which constitutive NF-κB activation is maintained in HIV-infected cells.

5) HIV-infected cells are more sensitive to TNFα induced apoptosis than uninfected cells. Inhibition of NF-κB exacerbates TNFα cytotoxicity in both infected and uninfected cell lines while deliberate activation of NF-κB partially rescues cells from death.

6) Inhibition of constitutive NF-κB DNA binding, correlates with the activation of an apoptotic signaling pathway in HIV-infected cells suggesting that HIV-infected monocytic cell lines resist apoptosis by activating NF-κB.

These findings have allowed us to construct a model of NF-κB regulation in HIV-infected myeloid cells. HIV infection induces cytokine expression (IFNα/β and TNFα) which leads to PKR and IKK activation. These kinases lead to the degradation of IκBα and IκBβ and the constitutive activation of NF-κB. Constitutive NF-κB activation counters a persistent apoptotic signal in HIV-infected monocytic PLB-IIIB and U9-IIIB cells, maintaining cell viability. Upregulation of this pathway in monocytic cells from HIV-infected individuals could explain the resistance of these cells to HIV-induced apoptosis.

Chapter VII

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