NOTE TO USERS

Regulation of Nuclear Factor κB subunit c-Rel through phosphorylation by two IKK-related kinases, IKKε and TBK-1

By

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Your file Votre référence
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To my husband Rémi
For your love, patience, understanding,
Constant encouragement and support

To my father Danny
For the faith you have always had in me, that made me believe in myself
And for the gift you gave me to love science

To my sister Virginie
For your constant listening and encouragements
And for peeking your head through the door for my midnight greeting!

To my brother Kevin, my mother Elaine and my parents in law Claudette and Lucien
For their love, faith and support

ABSTRACT

The nuclear factor κB (NF-κB) transcription factors are key regulators of immunomodulatory genes regulation. NF-κB activity is regulated through the phosphorylation of inhibitory proteins (IκBs) by the IκB kinase (IKK) complex (IKK α/β/γ), leading to IκB degradation and NF-κB translocation to the nucleus where they promote transcription of immunoregulatory genes. Moreover, cRel and p65 activities are also regulated by direct phosphorylation of their transactivation domain. Recently, two IKK non-canonical homologues, IKKε and TBK-1 (TANK binding kinase-1) have been identified with functions distinct from the classical IKKα/IKKβ. TBK-1/IKKε trigger antiviral immunity through direct phosphorylation of the IRF3/IRF7 transcription factors, which are key regulators of the interferon response. Since IKKε modulates the activity of IRF3/IRF7, it is of interest to assess whether IKKε/TBK-1 also regulates the transactivation activity of NF-κB. Our hypothesis was that IKKε/TBK-1 modulates the activity of cRel by direct phosphorylation of its transactivation domain (TD). In this study, we demonstrate that IKKε and TBK-1 directly phosphorylate cRel *in vitro* and *in vivo*. Two of the three consensus sequences recognized by IKKε/TBK-1 in the cRel TD are directly phosphorylated by IKKε. cRel was translocated to the nucleus in cells expressing wild type versus kinase dead variant. The expression of IKKε increases c-Rel transactivation in reporter gene assays. Serine to alanine mutation was further used to characterize the function of this phosphorylation at the level of nuclear translocation and transactivation potential using immunofluorescence and reporter gene assay. Furthermore, co-expression studies revealed that IKKε and not the kinase dead variant is responsible for cRel degradation in a dose-dependent manner and this effect is partially reverted by proteasome inhibition. These results suggest a new level of regulation for cRel by direct phosphorylation by the IKK-related kinases IKKε/TBK-1.

RÉSUMÉ

Les facteurs de transcription κB (NF-κB) sont des régulateurs clés requis pour la modulation des gènes impliques dans la réponse immunitaire. L'activation de NF-κB est régulée par la phosphorylation de protéines inhibitrices IκBs (inhibitor of κB) qui séquestrent NF-κB dans le cytoplasme des cellules non activées. Cette phosphorylation est médiée par le complexe de kinases IKK α/β/γ (IκB kinase (IKK)). Ceci provoque la dégradation des IκBs libérant ainsi les sous-unitées NF-κB qui transloquent alors au noyau ou ils permettent la transcription de gènes régulant la réponse immunitaire. De plus, l'activité de cRel et p65 est également régulée par phosphorylation directe de leurs domaines de transactivation. Récemment, deux homologues non classiques des IKK - IKKε et TBK-1 (TANK binding kinase-1)- ont été identifiée avec des fonctions distinctes des IKKα/IKKβ classiques. TBK-1/IKKε engendrent une immunité antivirale via la phosphorylation directe des facteurs de transcription IRF3/IRF7, qui sont des régulateurs clé de la réponse interféron. Étant donne qu'IKKε module l'activité de IRF3/IRF7, il logique de vérifier si IKKε/TBK-1 pourraient également réguler l'activité transcriptionelle de NF-κB. Notre hypothèse est donc qu'IKKε/TBK-1 module l'activité de cRel par phosphorylation directe de son domaine de transactivation (TD). Dans cette étude, nous démontrons qu'IKKε et TBK-1 phosphorylent directement cRel *in vitro* ainsi *que in vivo*. Deux des trois séquences consensus reconnues par IKKε/TBK-1 qui se retrouvent dans le domaine de transactivation de cRel sont directement phosphorylées par IKKε/TBK-1. cRel transloque au noyau dans des cellules qui expriment IKKε de type sauvage versus la forme mutée de la kinase laquelle est inactive (K38A). L'expression d'IKKε augmente la transactivation de c-Rel dans des essais de gène rapporteur (essais luciferase). L'étude de divers mutants de serine en alanine par analyse de translocation nucléaire de c-Rel et essais kinase in vitro a permis de conclure que plusieurs sites de phosphorylation sont impliques dans la phosphorylation par IKKε/TBK-1. De plus, par études de co-expression nous observons que IKKε de type sauvage et non le variant sans activité kinase est responsable de la dégradation de c-Rel de façon dose-dependent, un phénomène qui est inhibe partiellement par l'inhibition du proteasome. Ainsi, les

résultats obtenus lors de cette étude suggèrent un nouveau niveau de régulation de c-Rel par phosphorylation directe par les kinases reliées aux IKK: IKKε et TBK-1.

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PREFACE

In accordance with the guidelines for the thesis preparation, I have chosen to present the results of my research in classical form. In Chapter I there is the general introduction and a detailed review of the literature on the subject followed by the Material and Method section in Chapter II and the results section in Chapter III. Results are discussed in Chapter V and references are listed in Chapter IV.

The candidate is responsible for the most part of the work presented in this thesis, nevertheless a few colleagues have participated in some experiments. I would like to acknowledge Dr. Sonia Sharma for the cloning of some c-Rel constructs (GST-cRel 422-588, GST-cRel peptides A, B and C) as well as for the idea of generating smaller peptides. She has demonstrated that c-Rel was phosphorylated by IKKε in peptides A and C and that IKKβ phosphorylated peptide B *in vitro*. The results presented in this thesis were generated by the candidate and thus confirmed the results obtained by Dr Sharma.

Dr Nathalie Grandvaux is responsible for figure 20, panel C and she contributed considerably to many other experiments that are shown or not in this report. She has also provided me excellent supervision during the achievement of this project.

Dr Qiang Sun is responsible for the purification of the recombinant TBK1 kinase and for kinase assays showing phosphorylation of c-Rel TD by TBK-1 (data not shown).

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

aa	amino acid
AP-1	activated protein-1
BRET	Bioluminescent Resonance Energy Transfer
cAMP	Cyclic AMP
CBP	CREB-binding protein
CKII	Caseine kinase II
CHUK	
CREB	cAMP response element binding protein
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
DTT	dithiothreitol
EDTA	ethylenediamine tetra-acetic acid
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal-regulated kinase
FITC	
GM-CSF	Granulocyte/macrophage colony stimulating factor
GFP	green fluorescent protein
GST	Glutathione S-transferase
HIV-1	Human immunodeficiency virus-1
HHV-8	Human Herpes Virus-8

HTLV-1	Human T cell leukemia virus-1
IκB	Inhibitor of κB
IKK	IκB kinase
IKKAP1	IKK-associated protein-1
IL	Interleukine
IPTG	isopropyl-beta-D-1-thiogalactopyranoside
IRAK	IL-1 receptor associated kinase
IRF	Interferon regulatory factor
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
LT-β	Lymphotoxin β
LZ	Leucine zipper
MAPK	mitogen-activated protein kinase
NAK	NF-κB activating kinase
NEMO	NF-κB essential modulator
NES	Nuclear export signal
NF-AT	nuclear factor of activated T cells
NF-κB	Nuclear factor κB
NIK	NF-κB-inducing kinase
NLS	nuclear localization signal
PBS	phosphate buffered saline
PCR	polymerization chain reaction
PEST	proline, glutamic acid, serine, threonine rich domain
PKC	protein kinase C

RHD	Rel homology domain
RNA	Ribonucleic acid
SCID	Severe combined immune deficient
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sp-1	stimulatory protein-1
TAD (or TD)	transactivation domain
TANK	TRAF family member-associated NF-κB activator
TBK-1	TANK binding kinase-1
TBP	TATA-binding protein
TNF	tumor necrosis factor
TRAF	TNF receptor-associated factor
WT	wild type

CHAPTER I

GENERAL INTRODUCTION

1. Introduction to NF-κB

The nuclear factor κB (NF-κB) proteins are transcription factors that play a central role in the life cycle of the cell, protecting against pathogens. Indeed, those transcription factors are essentials to regulate the expression of numerous genes that are involved in immunity, inflammation, cell survival, cell proliferation, stress responses, embryonic development as well as apoptosis (7, 54, 60, 132). NF-κB proteins are often referred to as central mediators of the immune response.

The NF-κB family of transcription factor is evolutionary conserved (63). In mammals, it is composed of 5 members (figure 1), namely Rel (c-Rel), RelA (p65), RelB, NF-κB1 (p50 and its precursor p105) and NF-κB2 (p52 and its precursor p100). In Drosophila *maganogaster*, three Rel proteins have been identified, which are named Dorsal, Dif and Relish (figure 1) (44, 80, 167)

1.1. Structure of NF-κB

The NF-κB proteins are structurally related and share a highly conserved amino-terminal 300-amino-acid region, called the Rel Homology Domain (RHD), that mediates DNA binding, dimerization and association with the inhibitor of κB proteins IκBs (figure 1). c-Rel, p65 and RelB also contain a transactivation domains (TD) in their carboxy-terminal region that is essential to enhance the transcription of target genes. These TDs interact with proteins from the transcriptional machinery such as TBP (TATA-Binding Protein) and TFIIB (Transcription Factor IIB) as well as with transcriptional coactivators such as p300 and CBP (cyclic-AMP-response element (CREB) binding protein) (16, 133, 154, 160, 188). The transactivation domains of p65 and c-Rel are subject to regulatory phosphorylations. This regulation mechanism will be the main focus of this thesis. The uniqueness of the p50 and p52 subunits is that they lack such a transactivation domain. p50 and p52 can bind to DNA through κB binding sites, but need to heterodimerize with a subunit that harbors a transactivation domain to positively regulate gene expression. Otherwise p50/p50 or p52/p52 homodimers act as transcriptional repressor (119).

The NF-κB proteins also carry a nuclear localization signal (NLS) that is hidden in the inactive state of NF-κB through binding with the inhibitor of κB (IκBs). The crystal structure of IκBα/NF-κB complexes revealed that the amino-terminal ankirin

repeats of IκBα (figure 1) is positioned next to the NLS of p65 and stericly impedes its binding to the nuclear transporter proteins of NF-κB (77, 82).

1.2. The IκB family

To date, there are seven known mammalian members of the IκBs, which are IκBα, IκBβ, IκBε, IκBζ, BCL3 and the precursors IκBδ/p100 and IκBγ/p105 with the predominantly encountered form being IκBα (192) and reviewed in (86). The IκB proteins function as inhibitory proteins while BCL3 functions more as a transcriptional repressor. In Drosophila, there are only two known members of the IκB family, designated as Cactus and the homologue of p100 and p105, Relish (figure 1) (44). The IκBs proteins are composed of multiple ankirin repeats that are composed of a repeating sequence of 30-33 amino acids found in the ankirin protein. These repeats are involved in protein-protein interaction and bind to the RHD of NF-κB (60).

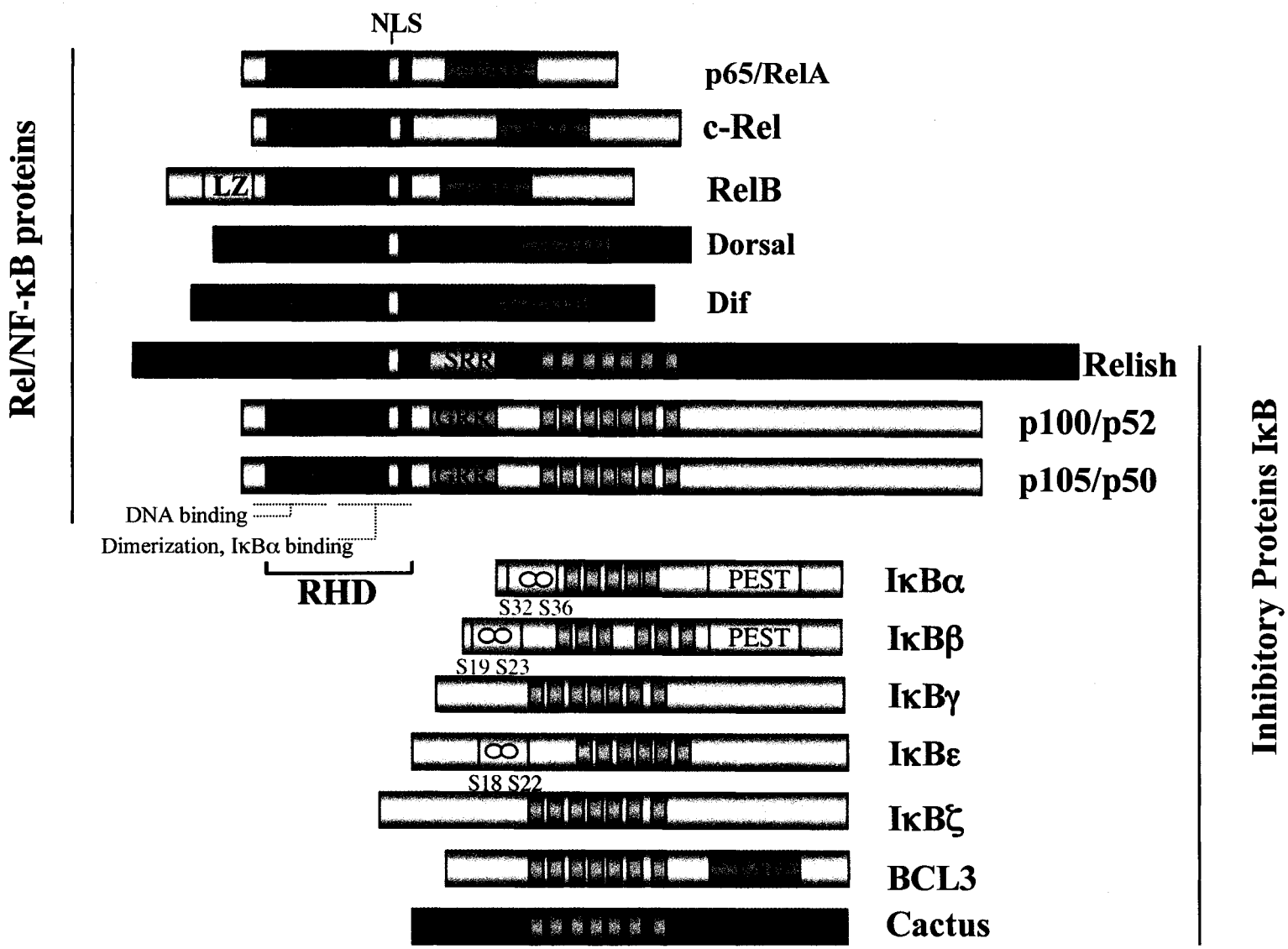
2. NF-κB subunits distribution, function and knockout mice phenotypes

The NF-κB subunits have various distributions throughout mammalian tissues. p65 and p50 are widely expressed in most cell types, whereas c-Rel expression is restricted to hematopoietic cells and RelB is found in some lymphoid tissues, dendritic cells and B cells (24, 60). Thus, although NF-κB subunits are structurally similar, their role will vary considerably due to the variation in their expression profile. Knockout and transgenic mice studies have brought up important information on the function of the different NF-κB subunits in the regulation of various aspects of the immune response, particularly in lymphocyte activation and innate immunity (24, 55). During the immune response, lymphocytes can be activated by foreign antigens, cytokines or growth factors produced by other stimulated cells thus triggering proliferation, differentiation or, in more drastic cases, apoptosis. Immune response analysis of knockout or transgenic mice revealed that all of these processes are defective or deregulated. For instance, B and T cells are able to develop as wild type cells when they lack c-Rel, p50 or p65 but they are showing various defects in lymphocyte activation, proliferation, cytokine production or immunoglobulin isotype switching (40, 92, 157). Furthermore, the immune response raised against several pathogens also show important defects in the absence of some

Figure 1. Rel/NF-κB and IκB family

The Rel/NF-κB family is composed of 5 members in mammals and 3 in Drosophila. These NF-κB proteins are characterized by a Rel homology domain (RHD), which contains the dimerization domain, the nuclear localization signal (NLS), which controls the translocation to the nucleus, and the DNA binding domain. RelA, c-Rel and RelB as well as Dorsal and Dif proteins harbor a transactivation domain TAD in their c-terminal portion, which is responsible for the transcriptional capacity of Rel/NF-κB. p100/p52 and p105/p50 act as NF-κB proteins but also as IκB proteins. Their c-terminal domains contain ankyrin repeats giving them the possibility to behave as IκB and impede nuclear translocation of NF-κB dimers. The maturation of these proteins is required for them to act as NF-κB. Proteolytic processing of these factors lead to the cleavage of the C-terminal end containing the ankyrin motifs. This leads to the generation of p50 and p52 that act as NF-κB through interaction with other NF-κB members.

The inhibitor of NF-κB (IκB) family contains 8 members in mammals including p100/p52, p105/p50 and 2 members in Drosophila: Cactus and Relish. Relish is the homologue of p100 and p105 with the exception of the serine rich region (SRR) instead of a glycine rich region (GRR), which are important regions for the co-translational processing of p105 to p50 and post-translational processing of p100 to p52 (106, 155) The IκB proteins are characterized by their glycine rich region (GRR) as well as by ankyrin repeats , which mediate protein-protein interaction with the IκB binding site into the RHD of the NF-κB proteins and mask the NLS of NF-κB proteins thus impeding their nuclear translocation. In addition, BCL3 contains a transactivation domain, which give transcriptional activities to this protein when complexed with either p50 or p52.


Rel/NF-κB proteins
NLS
p65/RelA
c-Rel
LZ
RelB
Dorsal
Dif
SRR
Relish
p100/p52
p105/p50
DNA binding
Dimerization, IκBα binding
RHD
PEST
IκBα
S32 S36
PEST
IκBβ
S19 S23
IκBγ
IκBε
S18 S22
IκBζ
BCL3
Cactus
Inhibitory Proteins IκB

NF-κB subunits, particularly in homozygous disruption of c-Rel, p50 or RelB (reviewed in (54)).

Thus, NF-κB subunits are differentially expressed and have been shown to have diverse role in innate and acquired immune response. Following are some specifications about each of the NF-κB subunits regarding their distribution and major functions according to knockout and transgenic mice studies.

2.1. p65/ RelA / NF-κB1

The p65 subunit is the most common subunit that harbors a transactivation domain and thus has the potential to activate transcription. *In situ* hybridization showed that p65 is expressed in all cell type from embryonic tissues with higher expression in the thymus (183). Therefore, it is not surprising that mice lacking p65 exhibit a profound abnormal phenotype. Indeed, *p65 -/-* mice die between day 15 and 16 of gestation due to apoptosis-induced liver degeneration (11, 12). None of the other members of the NF-κB family are able to compensate for the loss of p65, indicating that this developmental role is specific to p65. Hepatocytes from *p65-/-* embryos appear to be more prone to the cytotoxic effect of TNF-α, indicating that p65 play a role in the protection from TNF-α induced apoptosis (39). p65 seems to also have an anti-apoptotic role in fibroblasts and macrophages. (11). In fact, it is known that p65 promotes the expression of both survival and anti-apoptotic genes. Contradictorily, p65 can promote apoptosis in certain cell type in response to particular signals. Recent findings effectively suggest p65 as a major regulator of apoptosis of the immune system. (Reviewed in (94)).

To overcome the lethality of p65 knockout and to further analyze the role of p65 in immunity, SCID mice (Severe Combined Immune Deficient) reconstituted with E13 p65-/- fetal hepatocytes were generated (40). These studies indicate that p65 is dispensable for the development of lymphocytes but is required for their proliferation as well as for immunoglobulin isotype switching.

2.2. RelB

RelB is restricted to lymphoid tissues such as to specific regions of the thymus, lymph nodes and Payer's patches as well as to dendritic cells and B lymphocytes. RelB

was shown to be required for both acquired and innate immunity. Indeed, defects in lymphocyte activation, CD8+ cytotoxicity and macrophage-mediated immune response to a variety of pathogens have been reported (23, 185). Defects in humoral immunity of *relb* -/- mice arise due to impaired antigen presentation by CD4+ T cells. RelB seems to be dispensable for lymphoid cells development but is believed to be involved in the development of dendritic cells in the bone marrow as well as UEA-1+ medullary epithelial cells in the thymus. Mice lacking RelB develop normally until about day 10 post-natal. After this time, they start to show several abnormalities such as T cell inflammatory infiltration of various organs, thymic atrophy, splenomegaly due to extramedullary hemopoiesis and T cell-dependent myeloid hyperplasia. The inflammatory pathological phenotype might be a consequence of the deficit in thymic and splenic dendritic cells, which would lead to ineffective auto-reactive thymocytes and T cell destruction (21, 184).

2.3. c-Rel

This subunit is the main subject of this thesis and will be described into more details. The following information on c-Rel regarding its expression and function was collected from knockout studies. Although similar to the other members of the family with related functions, c-Rel has its particularities (reviewed in (110)). The expression of c-Rel is restricted to hematopoietic cells including T and B lymphocytes, monocytes, granulocytes and erythroid cells. (27, 54). The c-Rel/p50 heterodimer is the predominant dimer in mature B cells, where it is found constitutively active in the nuclei as a consequence of mitogen stimulation (66). Based on knockout studies, c-Rel is not essential for the development of hematopoietic cells, which are present in a normal number with typical expression of cell surface markers such as Igκ and IL-2Rα chain. However, c-Rel appears to play an important role in the induction of a proper response to mitogen stimulation. Indeed, B and T cells from c-Rel knockout mice are unable to proliferate in response to most mitotic stimuli. Studies have demonstrated that c-Rel plays an essential role in the production of cytokines such as IL-2, IL-3 and GM-CSF (Granulocyte-Macrophage Colony-Stimulating Factor) by T cells (55, 56, 92).

c-Rel -/- B cells also failed to proliferate in response to anti-IgM stimulation, whereas p50 -/- B cells proliferated at the same rate than wild type B cells, demonstrating the essential role of c-Rel in B cell proliferation induced by IgM cross-linking (92, 157).

Mature B cells have constitutive c-Rel/p50 active dimers and these complexes can associate both with IκBα and IκBβ. Despite all the publications that relate information on the binding of c-Rel with IκBα, it is believed that in pre-B cells or T cells, c-Rel/p50 heterodimer associate mainly with IκBβ (112, 172). Thus, signals leading preferably to IκBα degradation (like B cell receptor engagement) would activate c-Rel/p50 only in mature B cells. Accordingly, in addition to the rapid and transient activation through IκBα degradation, mature B cells also have the possibility to sustain activation of c-Rel/p50 through degradation of IκBβ, because this inhibitor is thought to permit such a persistent activation as explained in section 9. In fact, stimulation of receptors such as the TNF family members CD40 and TNFR1/2, can lead to activation of c-Rel containing dimers through both IκBα and IκBβ degradation in mature B cells.

On the other hand, in mature T cells, c-Rel complexes are only bound to IκBβ as opposed to p65 complexes that are found to be associated with both IκBα and IκBβ. As for BCR, stimulation of TCR preferentially targets IκBα for degradation. However, costimulation of CD28 is a signal for rapid IκBβ degradation as well (68) (85). CD28 response elements (CD28RE) are found particularly in the promoters of IL-2 or IL-2Rα chain where c-Rel homodimers have the capacity to bind with very high affinity (76). Thus, during costimulation of the TCR with CD28, full T cell activation and high levels of IL-2 production are essentially dependent on c-Rel activation principally though IκBβ degradation.

In summary, studies of c-Rel -/- cells have demonstrated that this subunit is not essential for hematopoietic cell development but have an important role in their activation by mitogens such as LPS, IgM cross-linking and various pathogens, leading to cell proliferation and humoral response. Cells lacking c-Rel seem to compensate with the p65 subunit. However, those cells still have profound defects in lymphocyte activation indicating that c-Rel has a unique role in B and T cell activation.

2.1.1. Structure and sequence of c-Rel

As for other NF-κB members, c-Rel is composed of a Rel homology domain in the N-terminal portion of its amino acid sequence, which comprises a dimerization domain, a NLS as well as a DNA binding site (figure 1). In its C-terminal portion, c-Rel harbors a serine rich domain that functions as a transactivation domain. The transactivation domain has been mapped between amino acids 422 and 588 (117). The three-dimensional structure of c-Rel bound to the CD28RE of the IL-2 promoter has been determined, whereas the structure of the whole protein has yet to be resolved. Thus we have currently no information on the three-dimentional structure of the transactivation domain in relation with the whole protein (76).

The function of the transactivation domain has been studied in transgenic mice. Mice homozygous with a deletion of the C-terminal portion of c-Rel, which contains the transactivation domain, were generated (*c-rel* $^{\Delta CT/\Delta CT}$ mice) and showed normal dimer formation between c-Rel$^{\Delta CT}$ and other NF-κB subunits, showed normal DNA binding, but abnormal gene transcription (26). These results indicate that the transactivation domain is essential for proper functioning of c-Rel.

2.1.2. Implication of c-Rel in gene expression

As previously mentioned, c-Rel deletion in T cells blocks IL-2 expression suggesting that c-Rel is essential for the expression of this cytokine, which is a key growth factor for T cells (55, 92). For proper inducible gene expression, one of the first events that occur is the remodeling of chromatin across the promoter and enhancer region of the target gene. Indeed, c-Rel was shown to be required for chromatin remodeling across the IL-2 gene promoter, as well as for gene transcription in response to CD3/CD28 stimulation. However, c-Rel seems to be dispensable in response to PMA/Ca^{2+} ionophore/CD28 stimulation. This difference is probably due to the fact that PMA/ Ca^{2+} ionophore /CD28 is a much stronger inducer of IL-2 production than CD3/CD28 and probably involves other transcription factors such as NF-AT (142). The expression by T lymphocytes of other immunological factors such as IL-3, GM-CSF, IFN-γ and IL-2 receptor α chain in is also dependent on c-Rel transcription factor (56, 70, 111, 176). Another gene regulated by c-Rel is the IRF4 gene, which is a transcription factor that is

specific to immune cells and that is regulated at the transcriptional level (158). Our group has demonstrated that in the promoter region of the IRF4 gene, there is a κB binding site where c-Rel, p65 and p50 bind a well as a CD28RE site where c-Rel, NF-AT and p50 but not p65 bind.

In B cells, c-Rel is important for their survival and cell cycle progression, due to the fact that c-Rel -/- BCR stimulated B cells fail to produce cycline D3 and cycline E. The absence of c-Rel leads to defective B cell survival due to impaired expression of cytokines such as IL-6, IL-10 and IL-15, which are important in this process (74, 175, 176). Moreover, c-Rel has the potential to promote the expression of anti-apoptotic genes like Bcl-xL, Mcl-1 and the Bcl-2 homologue Bfl-1 (98, 131, 199).

Professional antigen presenting cells achieve their role in the protection against many pathogens principally through the production of IL-12. c-Rel is essential for IL-12 expression in TLR stimulated macrophages and dendritic cells. Indeed, in macrophages, the expression of IL-12 subunit p40 in response to LPS and/or IFN-γ has been shown to require c-Rel containing NF-κB complexes. In contrast, it seems that c-Rel is essential for IL-12 subunit p35 but not p40 in dentritic cells. The nature of differential requirement of c-Rel in IL-12 subunits production in macrophages and dentritic cells is currently (65, 150, 186).

Recently, c-Rel was found to be important for neuronal survival as its deletion abolishes the survival effect of the neuronal survival factor IL-1β which has the possibility in normal neuronal cells to activate c-Rel, p50 and p65 complexes through the degradation of IκBα and IκBβ (138). In contrast, p65 seems to be more involved in neurotoxic factor-induced cell death rather than survival factor-induced cell survival. However, the genes responsible for the difference between the survival effects of IL-1β through activation of c-Rel and the p65-induced neuronal cell death have not yet been identified as yet. Another recent report suggests that c-Rel is a transcription factor necessary for hippocampus-dependent long-term memory formation, thus giving an additional role for c-Rel in the regulation of genes expressed in neuronal cells (100).

Overall, genes expressed by c-Rel are implicated in cell survival, anti-apoptosis, cell cycle progression and proliferation of several hematopoietic cells. Therefore, it is not surprising that c-Rel can behave as an oncogene when its gene transcription function is

pathologically enhanced. Consequently explaining why c-Rel dysfunction can lead to several types of diseases, mainly hematopoietic cancers.

2.1.3. Role of c-Rel in cancer and other pathologies

c-Rel was first identified as the mammalian homologue to the avian reticuloendotheliosis retrovirus oncogene v-Rel (61). v-Rel has the potential to transform T and B cells into lymphomas as well as erythrocytes and dendritic cells. c-Rel, which is the product of a proto-oncogene, also has the potential to transform cells when aberrantly expressed and is found to have a role in many types of cancer such as leukemia, lymphoma and breast cancer (143). Reviewed in (62). c-Rel is an NF-κB member that is consistently associated with cellular transformation and is also implicated in the pathogenesis of the viral-induced adult T cell leukemia (ATL). This disease is triggered following the infection of T cells by the Human T cell leukemia virus-1 (HTLV-1). In about 5% of infected individual, the infection ends up to the clonal expansion of infected T cells and results in an acute and fatal form of leukemia, the ATL. It has been shown that c-Rel plays a major role in the development of this disease because of its overexpression induced by the viral protein Tax (95).

It has also been reported that c-Rel is an important factor involved in pulmonary inflammation and allergen-induced asthma. This is based on the observation that allergic reactions such as increase in eosinophils and IgE secretion that is generally observed in asthmatic patients, are dramatically decreased in c-Rel knockout mice challenged with inhalation of allergens (41). Moreover, c-Rel knockout mice also showed reduced development of symptoms of autoimmune encephalomyelitis (EAE) disease, indicating that c-Rel might also have a role in the development of autoimmune diseases (70).

2.4. p105 (p50)

The p50 protein results from the processing of the p105 precursor through the proteasome pathway, which consists of the cleavage of the IκB-like c-terminal end (107). As for p65, p50 is widely and constitutively expressed in most cell types. Even if p50 is a component of the majority of all NF-κB dimers involved in development and function of immune cells, its absence neither leads to lethality nor to defects in development.

However, p50-/- mice are more susceptible to bacterial infection (190). Stimulation of p50-/- B cells with anti-IgM induces NF-κB activation leading to B cell proliferation. This suggests that p50 is dispensable for B cell proliferation induced by anti-IgM stimulation, and NF-κB probably transactivates genes through p65 and c-Rel. However, the B cell response to LPS does not lead to NF-κB activation in absence of p50 thus impeding cell differentiation. LPS stimulation but not IgM cross-linking requires p50 suggesting that these two stimuli activate different dimer combinations and pathways (127) Furthermore, according to the low levels of IgA, IgE and IgG1 in the serum of these knockout mice, it was concluded that p50 is most likely implicated in immunoglobulin isotype switching. Surprisingly, p50 knockout mice are more resistant to viral infection. The anti-viral innate immune response, which is mediated through the production of IFN-β, is increased as compared to wild type mice, indicating that p50 homodimers play a role in the repression of the IFN-β gene. According to the studies performed with p50 knockout mice, the p50 subunit seems to be important for the immune response but in contrast to p65, is not required for development (157).

2.5. p100 (p52)

Processing of the p100 precursor by the 26S proteasome leads to the cleavage of the c-terminal IκB-like portion of p100 resulting in the p52 form of NF-κB. The amount of p52 that is present in cells is generally lower then p50, but can be greatly induced by specific signals. The expression of p52 is restricted to specific areas of lymphoid organs, just as RelB. p52 can also be found in the stomach epithelial cells (3). Therefore, it is not surprising that p52 is RelB's main partner. p52-/- mice show defects in their splenic microarchitecture, in lymph node formation and they also lack Payer's Patches. These defects lead to an impaired immune response (25, 48). Recent findings indicate that p100/p52 is also important for B cell development, proliferation, survival and differentiation (34, 35). Studies of transgenic mice with C-terminal truncation of p52 indicate that both p50 and p52 are important for the tight regulation of NF-κB to prevent overexpression of target genes.

NF-κB members share high similarities in their structure and function, it is therefore likely that they have the ability to compensate for each other in the single

knockout cells. This compensation might prevent certain phenotypes that could have been observed in multiple knockout NF-κB genes studies. To further investigate the roles of NF-κB members, multiple NF-κB knockout mice were generated and resulted in more sever defects compared to the single knockouts. For instance, in p50/p65-/- mice, the development of lymphocytes was impeded early in lymphopoiesis compared to the single p50 or p65 knockout that did not interfere of B or T cell development (40, 73, 157). On the other hand, hematopoiesis of double knockout of p50 and c-Rel was not affected compared to WT mice. However proliferation and survival of mature B cells in response to various stimuli was impaired, indicating that c-Rel and p50 regulate mature lymphocyte functions and activation (67, 139).

3. Target genes of the NF-κB transcription factors

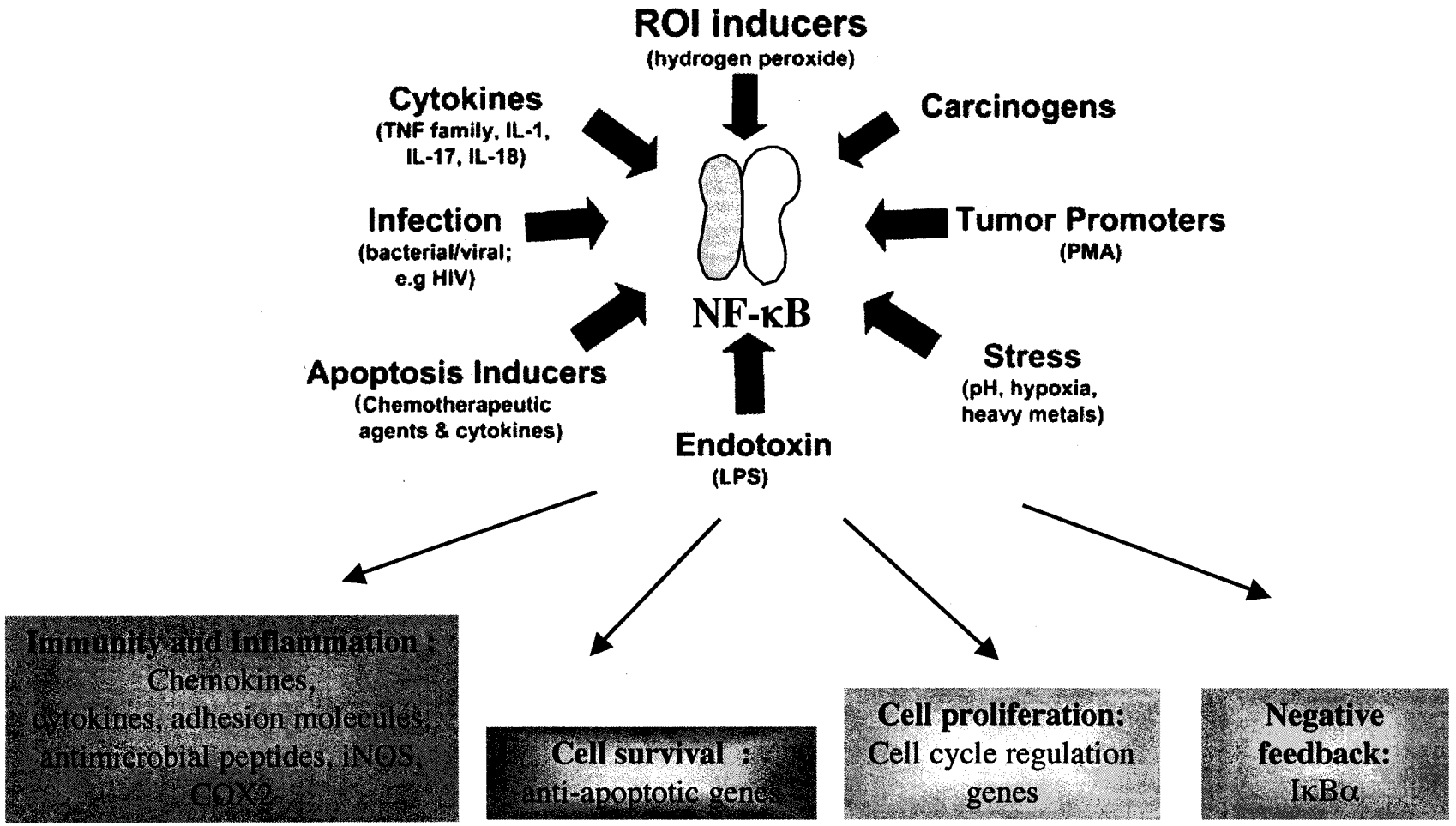
Among the numerous genes regulated by NF-κB, the majority encode proteins involved in the immune response. There are at least 27 different cytokines and chemokines, several receptors involved in immune recognition, proteins required for antigen presentation and receptors required for neutrophil adhesion and extravasation that are regulated by NF-κB (figure 2) (reviewed in (132)). The expression of NF-κB subunits like c-Rel, RelB and p105 (p50 precursor) is regulated by NF-κB itself (60, 177). NF-κB also has the potential to regulate the expression of its inhibitor IκBα, which acts as a negative regulator of NF-κB activity (58).

4. Pathologies associated with NF-κB dysfunction

The balance between anti-apoptotic and pro-apoptotic gene expression is mandatory to keep homeostasis in living cells. As the NF-κB transcription factors are central in the maintenance of this balance, any deregulation of the NF-κB function can lead to a wide variety of pathologies. Down-regulation of NF-κB has been associated with neuro-degenerative disorders such as Alzheimer disease (6). An unrestrained activation of NF-κB can lead to pathologic over-expression of survival, cell cycle and/or cell proliferation genes resulting in uncontrolled cell growth and cellular transformation, thus implicating NF-κB in a variety of cancers (114). Deregulation of NF-κB has also

Figure 2. NF-κB inducers and major induced genes

The NF-κB transcription factor can be activated by several stimuli such as various cytokines, infectious agents, carcinogens, oxidative stress or physical stress. Refer to table 1 for exhaustive list of inducers. Stimulation of the cell by these stimuli activates different signaling cascades that lead to NF-κB activation and depending on the inducer and the cell type, a wide variety of genes are transcribed. Genes that are regulated by NF-κB are implicated in many biological process including immunity and inflammation, cell survival, cell proliferation and also apoptosis. NF-κB can also regulate the expression of its own inhibitor, IκBα and thus negatively regulate itself. (Adapted from (53))



Adapted from Leukemia 16:1053-1068, 2002

been associated with acute and chronic inflammation disorders such as asthma, inflammatory bowel disease, arthritis and many others (8, 47, 128), reviewed in (169). In sum, NF-κB is an interesting target for the development of therapeutic strategies to overcome several diseases.

5. Inducers of NF-κB activity and related signal transduction.

In response to proinflammatory stimuli or pathogens, the activation of NF-κB occurs by a very complex process that is regulated at various levels and involves the coordination of numerous proteins. Regardless of the complex mechanism required to activate NF-κB, it remains a very rapid and effective process that leads to transactivation of essential genes implicated in the immune and inflammatory response. To overcome invading pathogens, the immune system must be prepared to trigger rapid signaling events that lead to the transcription of a wide variety of genes involved in the immune response. The NF-κB transcription factors are ideal to achieve such a rapid response as they can be rapidly activated without the need of de novo protein synthesis.

A clear indication of the central role of NF-κB transcription factors in the life of vertebrates is the fact that over 150 different stimuli have the potential to activate gene transcription through NF-κB. And in turn, due to the extended possibilities of association and regulation levels of these transcription factors, NF-κB was reported to contribute to transcription regulation of over 150 target genes. Inducers (Table 1) and target genes (figure 2) (53) can be categorized into general classes as described by Pahl et al (132). High numbers of pathways lead to NF-κB activation, and therefore will not be explained deeply for the purpose of this thesis. Most importantly related to this work are the Toll-like receptors (TLR) and T cell activation pathways that are linked to c-Rel and IKKε, which will be described in more details.

Table 1 Inducers of NF-κB activity

Category of NF-κB activity inducers	**Selected examples**
Bacteria	Enteropathogenic *E. coli*, *Helicobacter pylori*, *Mycobacteria tuberculosis*, *Salmonella typhimurium*, *Shigella flexneri*, *Staphylococcus aureus*
Bacterial products	Lipopolysaccharide (LPS), Muramyl peptides, Staphylococcus enterotoxins A and B, Toxic Shock Syndrome Toxin 1.
Viruses	Adenovirus, Cytomegalovirus (CMV), Epstein-Barr Virus (EBV), Hepatitis B and C virus (HBV and HCV), Human Herpes Virus-6 and 8 (HHV-6 and HHV-8), Human Immunodeficiency virus-1 (HIV-1), Human T cell leukemia virus-1 and 2 (HTLV-1 and HTLV-2), Influenza virus, Measles virus, Newcastle disease virus (NDV), Respiratory Syncytial Virus (RSV), Rhinovirus, Sendai Virus (SV).
Viral products	Double stranded RNA, Adenovirus E1A, , HBV: HBx, LHBs, MHBs, HCV core protein, HIV-1 gp160 and Tat, HTLV-1 Tax1, HTLV-II Tax2, Influenza virus hemagglutinin.
Cytokines	IL-1 IL-2, IL-12, IL-15, IL-17, IL-18, TNF-α, TNF-β, Type I interferon (IFNα, β).
Physiological stress conditions	Adhesion, hyperglycemia, hyperosmotic shock, liver regeneration, T-cell selection.
Physical stress	Ultraviolet irradiation (UV-A, B, C), γ-radiation.
Oxidative stress	Butyl peroxide, Hydrogen peroxide, Ozone, Pervanadate, Reoxygenation
Environmental stress	Chromium, cigarette smoke, Cobalt, Dicamba (herbicide, peroxisome proliferator), Lead, Nickel, Silicia Particles.
Therapeutic drugs	Azidothymidine (AZT), Etoposide, Methamphetamine, Taxol.
Modified proteins	Amyloid Protein Fragment (βA4), Oxidize Low Density Lipoprotein (LDL).
ER overload	Overexpressed proteins: Ig heavy chain, MHC Class I.
Receptor/Ligands	Antigen (IgM-Ligand), CD28-Ligand (B7-1), CD40-Ligand, CD4 ligand (gp120), Trail-receptor-1, 2 and 4-ligands.
Apoptotic mediators	Anti-Fas/Apo-1, Trail.
Mitogens, growth factors, hormones	Bone morphogenic protein 2 and 4, Human Growth Hormone, Insulin, M-CSF, Nerve Growth Factor, Platelet-Derived Growth Factor, TGF-α.
Physiological mediators	Anaphylatoxin C3a and C5a, Angiotensin II, Bradikinin, Heat shock protein 60, Leukotrien B4, PAF (platelet activating factor), Potassium, Thrombine.
Chemical agents	Calcium inonophores, Calyculin A, Con A, Cycloheximid, Okadaic acid, PHA, phorbol ester, PMA, Tunicamycin.

Updated adaptation of Pahl, HL *Oncogene* (1999) 18, 6853-6866 (132)

3.1. TLR3 and TLR4-mediated NF-κB activation: Response to virus and bacterial infection

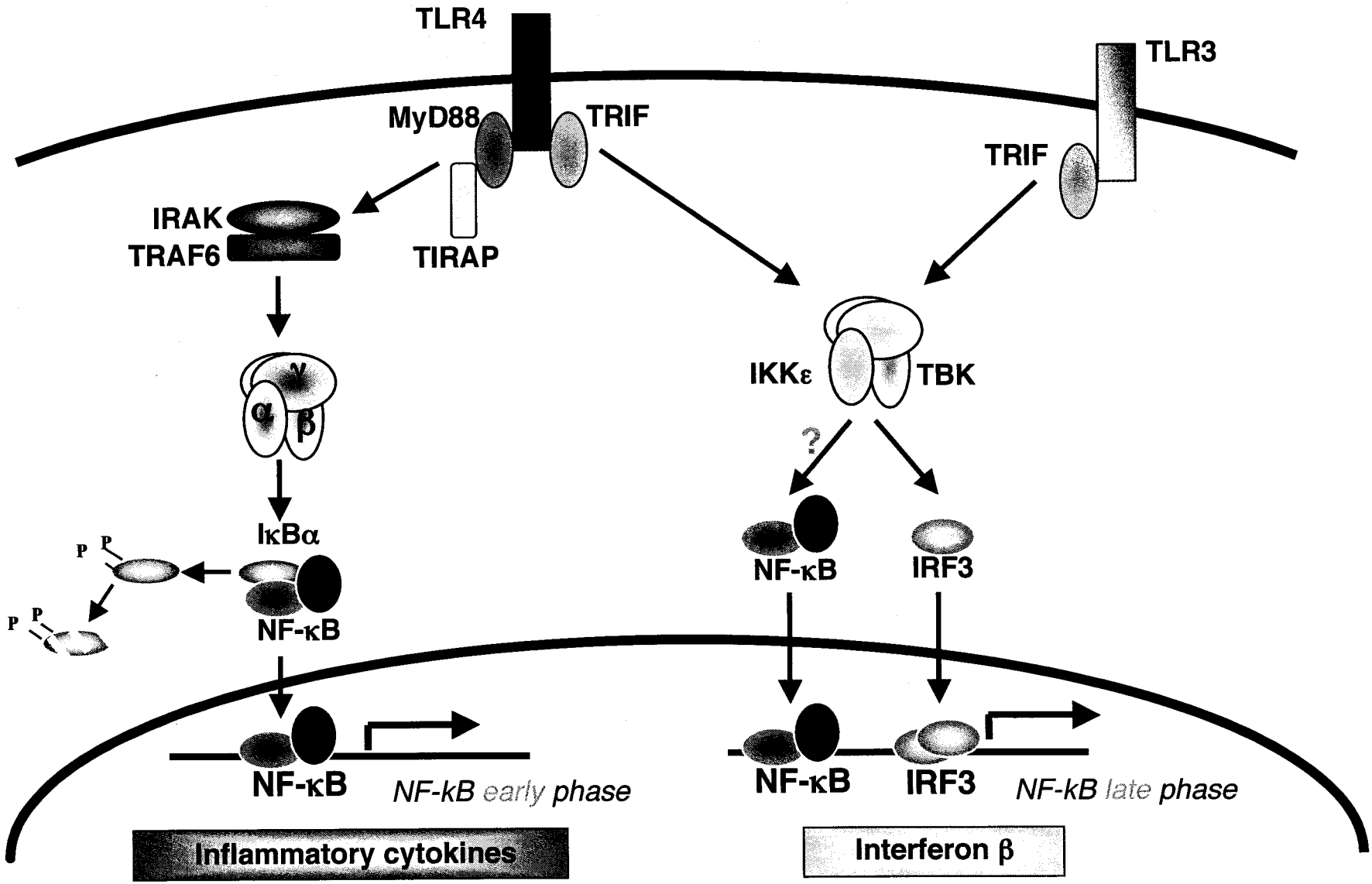
NF-κB is a central mediator of the immune response against a wide variety of bacteria and viruses. Through sensing specific pathogen compounds that are recognized by a family of receptors called Toll-like receptors (TLRs), the cell can activate signaling pathways ultimately leading to NF-κB dependent gene expression. TLR signaling can be mediated through stimulation of multiple receptors, which will involve several downstream signaling molecules that will lead to different patterns of gene expression mediated by several transcription factors, including NF-κB. This will lead to the activation of the innate immune response as well as the antigen-specific acquired immune response. To date, 11 TLRs have been identified in mammals and they appear to have a wide variety of ligands mainly characterized as molecular compounds coming from various pathogens (for recent review see (1)).

TLR stimulation first triggers the association of adaptor proteins to the TIR domain (Toll/IL-1R domain) of the receptor. Downstream signaling varies depending on which TLR is stimulated and specific TIR-domain-containing adaptor molecules are associated with specific TLR. Stimulation of TLR by any stimulus will ultimately lead to NF-κB activation through two particular pathways, which are either dependent or independent of the adaptor protein MyD88 (Myeloid differentiation primary-response protein 88).

Stimulation of TLR4 by LPS can trigger signalization through both MyD88-dependent and independent pathways, whereas TLR3 stimulation by dsRNA is exclusively independent of MyD88. The MyD88-dependent pathway triggered by TLR4 stimulation requires the recruitment of the adaptor protein TIRAP (TIR-domain-containing adaptor protein; also known as MAL (MyD88-adaptor-like protein)), while the MyD88-independent pathway recruits TRAM (TRIF-related adaptor molecule, also known as TICAM2 (TIR-domain-containing molecule 2)) and TRIF (TIR-domain-containing adaptor protein inducing IFNβ; also known as TICAM1 (TIR-domain-containing molecule 1 (Figure 3) (170). Studies have shown that in MyD88-deficient macrophages, the activation of NF-κB is completely blocked in response to stimulation of TLR2 with its proper ligand, but is delayed when TLR4 is stimulated with LPS,

Figure 3. NF-κB activation through TLR3 and TLR4 signaling through the classical IKK complex and the IKK-related kinases

TLR3 and TLR4 can be stimulated by dsRNA and LPS respectively and lead to signaling events that converge to NF-κB activation. When TLR4 is stimulated, adaptor proteins MyD88 or TRIF are recruited to the TIR domain of the receptor through binding with the TIR domain in the adaptor proteins. MyD88 is the adaptor protein that is essential for the signaling leading to early activation of NF-κB and to the production of inflammatory cytokines. TIRAP/Mal is another TIR domain-containing protein that associates with MyD88 in the downstream signaling of TLR4, as well as TLR2 (not shown). TLR3 signaling is independent of the adaptor protein MyD88 and recruits TRIF to the cytoplasmic TIR domain of TLR3. The MyD88-independent signaling leads to the activation of IRF3 through phosphorylation by IKKε and TBK1. The MyD88-independent TRIF-dependent pathway is also downstream of TLR4 and is believed to lead to the late phase of NF-κB activation. Both NF−κB and IRF3 are required for the production of IFNβ. However, the activation of NF-κB by the IKK-related kinases IKKε and TBK-1 is not completely understood (adapted from (170)).


TLR4
TLR3
MyD88
TRIF
TRIF
IRAK
TRAF6
TIRAP
IKKε
TBK
?
IκBα
NF-κB
NF-κB
IRF3
NF-κB
NF-κB
IRF3
NF-kB early phase
NF-kB late phase
Inflammatory cytokines
Interferon β

suggesting that the MyD88-independent activation of NF-κB involves a later phase of activation (88). NF-κB-mediated gene expression independent of MyD88 is suggested to be restricted to interferon and some genes known to be interferon-stimulated genes. Conversely, inflammatory cytokines gene expression is dependent on MyD88 (Figure 3). Therefore, NF-κB is rapidly activated in response to TLR4 stimulation by the MyD88-dependent pathway, thus implicated in the early phase of NF-κB activation and provides the cell with rapid production of inflammatory cytokines. This MyD88-dependent pathway leading to rapid activation of NF-κB and expression of inflammatory cytokines is also used by TLR1, TLR2, TLR5, TLR6, TLR7 and TLR9 (1). By the MyD88-independent pathway, TLR4 stimulation by LPS would activate the late phase of NF-κB as well as the IRF3 transcription factor and this would lead to the production of interferon and interferon-stimulated genes.

The TLR3 receptor signalization pathway leading to NF-κB and IRF3 activation is independent of MyD88 and requires the recruitment of a different adaptor molecule containing a TIR domain, called TRIF (TIR domain containing adaptor protein inducing IFN-β) (191) (figure 3). Double stranded RNA (dsRNA), a by product of virus replication, or synthetic dsRNA polyinosinic-polycytidylic acid (poly I:C) stimulate this receptor. This particular MyD88-independent/TRIF-dependent pathway will lead to the activation of NF-κB and IRF3, which are both transcription factors required for the expression of interferon-β and some IFN-stimulated genes. IRF3 is a key transcription factor that the cell activates in response to virus infection to produce type I interferons (IFNα and IFNβ) to induce an anti-viral state. Our group has recently demonstrated that IRF3 is activated through direct phosphorylation by IKKε and TBK-1 (159).

3.2. T cell activation: TCR stimulation and CD28 costimulation leading to NF-κB activation

In the adaptive immune response, T and B lymphocytes are activated by stimulation of the T-cell antigen receptor (TCR) or B cell receptor (BCR) and by costimulatory signals. Antigen-presenting cells (APC) present foreign antigens on MHC (major histocompatibility complex) molecules, which are recognized by the TCR or BCR on lymphocytes. Monoclonal antibodies to TCR or to CD3, which is a receptor-

associated molecule, can trigger the activation of T cells by mimicry of the antigen recognition from APC (122). Stimulation of TCR or BCR as well as costimulation signals lead to the activation of NF-κB, albeit through different signaling pathways. The signaling events upstream of IKK, IκB and NF-κB are not completely understood (Reviewed in (144)). For the purpose of this thesis, I will mainly focus on the TCR signaling events and CD28 costimulatory signal, where c-Rel is known to be implicated in the regulation of NF-κB-dependent gene expression, particularly for IL-2 secretion (20).

Following TCR stimulation, one of the major cytokine produced by activated T cells is IL-2. This cytokine allows long-term proliferation of T cells through autocrine and paracrine manner. However, without CD28 costimulatory signal, the activation of NF-κB is modestly activated and the amount of IL-2 produced is relatively low (reviewed in (106)). Therefore, for optimal T cell activation and efficient IL-2 secretion, both TCR and CD28 stimulation are required. The CD28 response element (CD28RE) is found in the promoter region of several lymphokines such as IL-2, IL-3, GM-CSF and IFN-γ (33, 49, 50, 179). Importantly, all of these cytokines are not produced properly in c-Rel -/- mice activated T cells (56, 92). c-Rel is known to bind and activate the CD28RE found in the promoter of the IL-2 gene (57). Thus, the CD28 costimulation increases IL-2 secretion of activated T cells dependently of c-Rel and is essential for complete T cell activation.

It is believed that the kinetic of T cell activation includes early and late events (Reviewed in (81)). The immediate and early phases of T cell activation require NF-κB-mediated transactivation mainly through p65, as well as NF-AT and AP-1 for IL-2 production. In the late phase of T cell activation, at least two transcription factors are responsible for the sustained expression of IL-2, including c-Rel and Oct2. The phenotype of c-Rel knockout T cells clearly demonstrate that c-Rel is essential for IL-2 production (56, 92, 111) as compared with Oct2-/- T cells where IL-2 production is not abrogated (36). Thus, it is believed that c-Rel is the most important transcription factor required for sustained IL-2 production in the late phase of T cell activation (Reviewed in (81)).

IKKε was shown to be part of a novel PMA-inducible IKK complex following PMA treatment, which is a compound that mimic TCR stimulation through activation of PKCs, suggesting that IKKε is involved in PMA/CD3 induction of NF-κB (134). Moreover, and IκBα ser32/36 kinase activity can be immunoprecipitated with anti-IKKε antibody when jurkat cells are activated by anti-CD3/CD28 antibodies (135). Taken together, the information from the literature suggests strongly that IKKε is a PMA and T cell activation-dependent IKK implicated in NF-κB activation.

3.3. Cytokine-induced NF-κB activation

Several proinflammatory cytokines such as TNF-α and IL-1 activate NF-κB. These inducers activate the classical pathway of NF-κB as described in section 7.1.1 (figure 4). The activation of NF-κB by these inducers leads to the expression of NF-κB–dependent genes that are involved in the inflammatory response (Reviewd in (59)).

3.4. Physical stress and chemical agents (mitogens, UV irradiation)

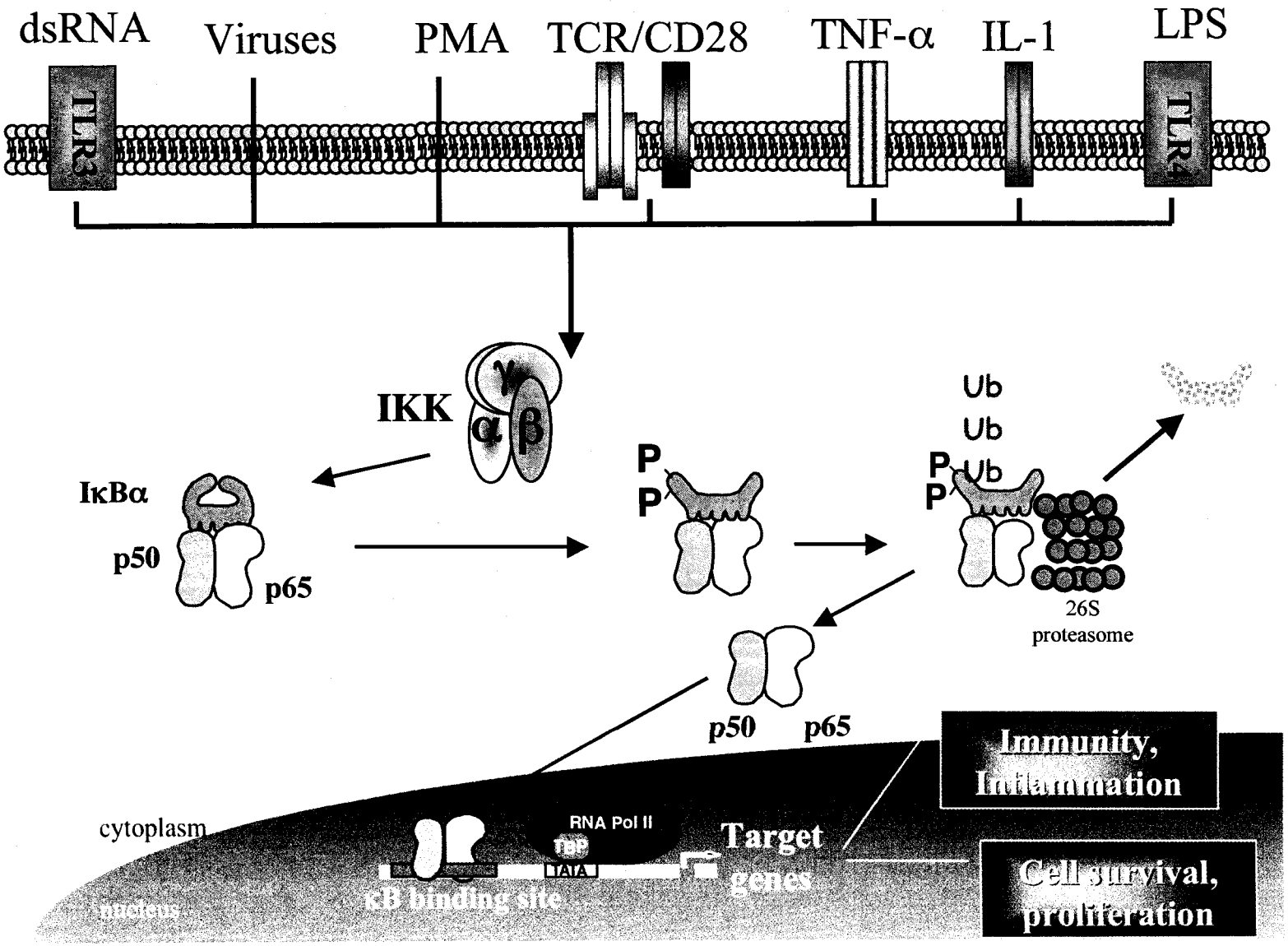
NF-κB is not only central to the immune response, but is also known to be a more general regulator of various cellular stress responses, like physical or oxidative stress. In the presence of an imminent stress event, the activation of NF-κB informs the cell and enables it to react rapidly through the expression of genes that will protect the cell against the stress situation and will either prevent the cell's destruction or trigger apoptosis when the situation is too critical (reviewed in (60)). NF-κB can also be activated by environmental stress such as cigarette smoke, herbicides, pesticides and other chemical as well as various therapeutic drugs (table 1) (132).

6. Mechanism of NF-κB activation

The NF-κB proteins act as dimers that bind to κB binding sites found in the promoter and enhancer regions of target genes. Subunits can form homodimers or heterodimers with one another, except for RelB, which has never been found as an homodimer. The most frequently encountered form of active NF-κB is a heterodimer of p65 associated with p50 or p52. The precursor proteins p105 and p100 are processed

Figure 4. The classical pathway to NF-κB activation

NF-κB can be activated through two main pathways: the classical (or canonical) and the non-classical (non-canonical) pathways. Various inducers such as dsRNA, viruses, PMA, TCR stimulation and co-stimulation, TNF-a, IL-1 and LPS activate the classical pathway of NF-κB activation. This consists of the activation of the classical IKK complex composed of IKKα and IKKβ as catalytical subunits and the accessory subunit IKKγ. Activation of the IKK complex leads to the phosphorylation of the inhibitory protein IκBα that retains NF-κB dimers in the cytoplasm by masking their NLS. Phosphorylation of IκBα is a signal for polyubiquitination and degradation through the proteasome pathway. Liberation of NF-κB dimers from the inhibitory protein allows nuclear translocation of NF-κB, followed by binding to κB binding sites in the promoter and enhancer regions of target genes. NF-κB responsive genes, such as genes implicated in the immune response, inflammation, cell survival and proliferation are then transcribed.


dsRNA
Viruses
PMA
TCR/CD28
TNF-α
IL-1
LPS
TLR3
TLR4
IKK
γ
α
β
IκBα
p50
p65
P
P
Ub
Ub
P
Ub
P
26S
proteasome
p50
p65
Immunity,
Inflammation
cytoplasm
RNA Pol II
TBP
TATA
κB binding site
Target
genes
nucleus
Cell survival,
proliferation

through proteasome-mediated proteolytic cleavage of the inhibitory domain and result into p50 and p52 subunits respectively. To become activated the NF-κB dimers first have to be freed from the IκBs. Once freed, the NF-κB dimers translocate to the nucleus and bind to κB binding sites, which are decameric sequences present in one or more copy in the promoter and enhancer regions of NF-κB regulated genes. Different dimer combinations have various binding affinities to the κB binding sites that share the following DNA consensus sequence 5'-GGGRNNYYCC-3' (R = purine, Y = pyrimidine, N = any nucleic acid) (reviwed in (86, 123)). With the recruitment of coactivators and the transcription machinery, NF-κB targeted genes will then be transcribed.

During the normal process of NF-κB activation, the upregulated target genes can lead to different outcomes like apoptosis or cell survival, depending mainly on the inducing signal. To achieve its important and diverse roles, the NF-κB transcription factors activity must be finely tuned. The precise mechanism for the specificity of NF-κB driven gene transcription is not fully understood. However, cell-type-specific expression of NF-κB members, various dimer combinations, diverse signaling pathways, different inhibitory proteins, copy number of κB sites in the promoter of target genes, variability in DNA sequences of κB sites, direct phosphorylation/dephosphorylation or acetylation of NF-κB subunits or histone tails, interaction with coactivators are all known levels of regulation that possibly occur to give specificity to the NF-κB dependent gene transcription (reviewed in (31, 59, 86)).

Two specific levels of regulation are of particular importance for this thesis. The first one is that NF-κB activity vary according to the inhibitory protein is bound to the dimer. Despite the fact that IκBα and IκBβ interact with various NF-κB dimers through similar affinity, they apparently behave differentially according to their capability to shuttle between cytoplasm and nucleus. Indeed, IκBα is able to easily shuttle NF-κB dimers from the cytoplasm to the nucleus and vice versa because it harbor both a nuclear localization (NLS) and nuclear export signals (NES) in its sequence. This NES enables newly synthesized IκBα to transfer nuclear NF-κB dimers back to the cytoplasm (136). Conversely, IκBβ lacks such a nuclear export sequence, thus retaining NF-κB dimers in the cytoplasm through hiding of the NLS of NF-κB proteins. Therefore IκBβ is able to

keep NF-κB dimers in the cytoplasm more efficiently than IκBα. Once freed from IκBβ, NF-κB activity would be more sustained due to the fact that IκBβ cannot take the nuclear dimers back to the cytoplasm. On the other hand, dimers bound to IκBα would be activated transiently because of the potential of IκBα to shuttle NF-κB dimers rapidly to the cytoplasm (172). These distinct properties of IκBs are one of the many levels of regulation of the finely tuned NF-κB activity. Among all the levels where NF-κB can be regulated, the direct phosphorylation of NF-κB subunits is another important step that will be focused for this thesis.

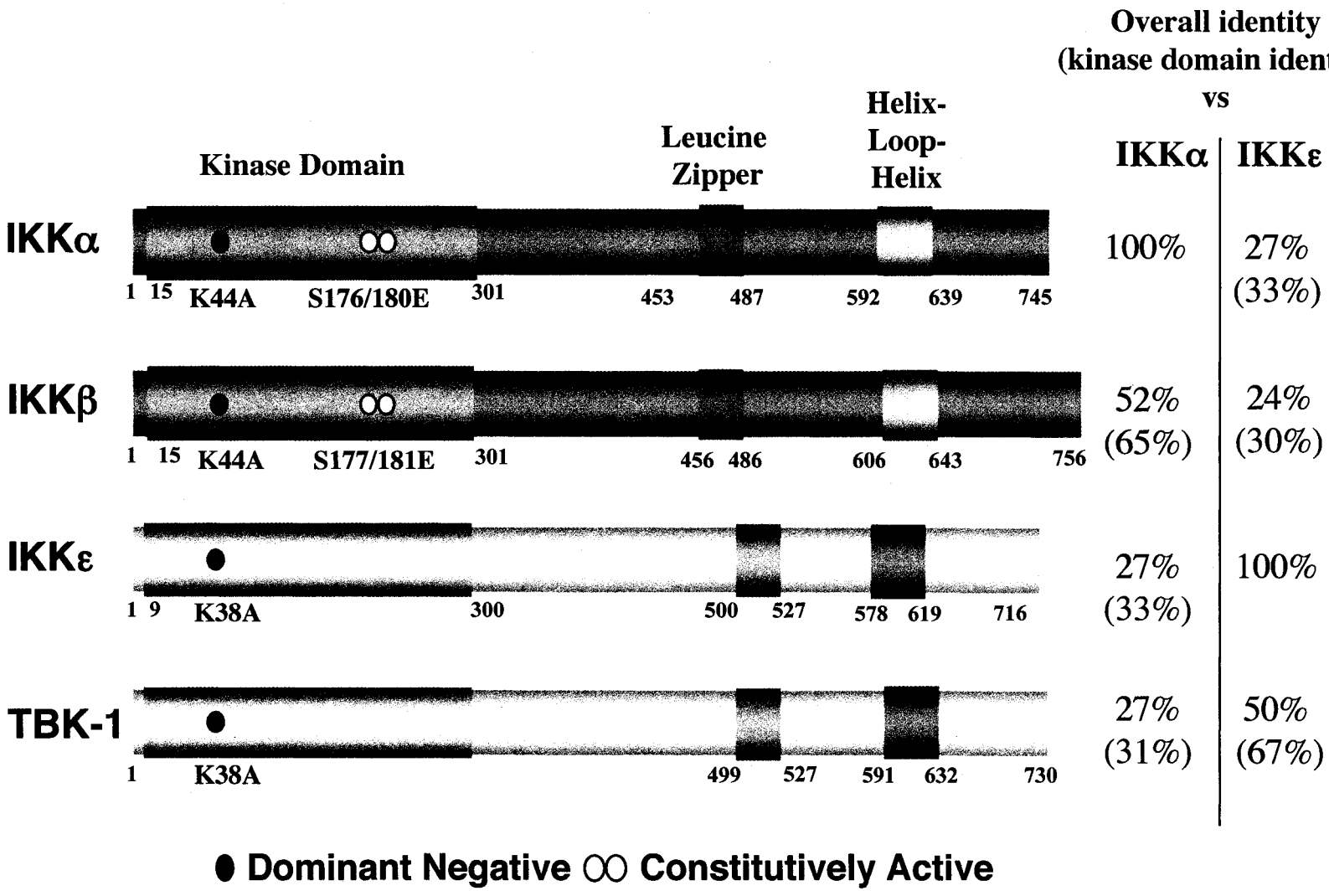
7. The IκB kinases (IKKs)

7.1. The classical IKKα/β/γ complex

Generally, most signals that trigger the activation of NF-κB can do so through the activation of a multimeric kinase complex, the IκB kinase (IKK). The IKK complex is composed of two catalytic subunits IKKα (IKK1 or CHUK (Conserved Helix-loop-helix Ubiquitous Kinase)) and IKKβ (IKK2), with an essential regulatory subunit, IKKγ (also known as NF-κB essential modulator (NEMO), IKK-associated protein-1 (IKKAP1) or 14.7 interacting protein (FIP-3)). Although IKKγ lacks kinase activity, it is essential for the activity of the complex as in the absence of IKKγ, no IKK or NF-κB activation is possible (86, 87). IKKα and IKKβ share 70% of similarity in their amino acid sequences and 50% identity. They both harbor an amino-terminal kinase domain, a helix-loop-helix and leucine zipper motifs responsible for protein-protein interactions (figure 5) (121, 195). They also have homology with the newly discovered IKK-related kinases IKKε and TBK-1, which are described in section 7.2. IKKα and IKKβ also contain an identical activation loop within their kinase domain (38, 109, 121). Two serine residues located in the activation loop are part of a MAP/MEK target consensus motif, Ser-X-X-X-Ser (X = any amino acid). Phosphorylation of S176/180 or S177/181 in those activation loops by upstream kinases is required to activate IKKα and IKKβ respectively. Mutation of those serine residues into alanine generates kinase-defective versions of IKKα and IKKβ (109, 121), whereas conversion of those serine into glutamic acid for phosphomimetic, produces constitutively active kinases (121) (figure 5). Both serines in the activation loop

Figure 5. The IκB kinase (IKK) family

The schematic representation of the classical IKKs (IKKα and IKKβ) and the non-canonical IKK-related kinases (IKKε and TBK-1) show homology in their structure with a N-terminal kinase domain and C-terminal leucine zipper motif as well as helix-loop-helix domains. All four kinases phosphorylate the inhibitor of κB protein IκBα. The classical IKKα and IKKβ share 52% overall identity to each other with 65% identity in their kinase domain and they phosphorylate serines 32 and 36 of IκBα.The non-classical IKKε and TBK-1 share 50% overall identity to each other with 67% identity in their kinase domain and they phosphorylate serine 36 of IκBα only. There is 30% identity in the amino acid sequence of the kinase domains of IKKε and TBK-1 when compared to IKKα and IKKβ (78). Mutation of amino acid at position 44 or 38 in the kinase ATP-binding pocket of the kinase domain of IKKα and IKKβ or IKKε and TBK-1 respectively, abolish the kinase activity and act as dominant negative mutants. Mutation of IKKα serines 176/180 into glutamic acid residue or of IKKβ serines 177/181 generate phosphomimetic mutants that are constitutively active kinases.


Overall identity
(kinase domain identity)
vs
IKKα
IKKε
Kinase Domain
Leucine
Zipper
Helix-
Loop-
Helix
IKKα
1 15 K44A S176/180E 301 453 487 592 639 745
100%
27%
(33%)
IKKβ
1 15 K44A S177/181E 301 456 486 606 643 756
52%
(65%)
24%
(30%)
IKKε
1 9 K38A 300 500 527 578 619 716
27%
(33%)
100%
TBK-1
1 K38A 499 527 591 632 730
27%
(31%)
50%
(67%)
● Dominant Negative ○○ Constitutively Active

are phosphorylated by upstream kinases in response to TNF-α or IL-1 stimulation (38). Moreover, several overexpressed kinases (reviewed in (86)), such as MEKK1 or NIK (97, 125), induce phosphorylation of both serines and activate IKKα and IKKβ.

Knockout studies with deleted IKK subunits revealed that IKKβ and IKKγ are essential for TNF-α or IL-1 activation of NF-κB through the canonical pathway, but IKKα is dispensable (103, 162). IKKβ defective mice show a phenotype similar to the p65-/- mice, which is lethal between days E12.5-E14.5 due to massive liver degeneration from TNF-α-induced apoptosis, thus suggesting a role for IKKβ in TNF-α stimulated activation of NF-κB (105, 173). IKKα-deficient mice exhibit abnormal development of skin and skeleton and showed only diminished response to NF-κB activation by TNF-α or IL-1 (75, 104, 171). IKKα/IKKβ double-knockout cells exposed to a variety of extracellular stimuli show a complete lack of IκBα phosphorylation and degradation (103). During NF-κB activation, the stimulation of IKK can lead to the activation of NF-κB by two major different pathways, namely the canonical and non-canonical pathways (141).

7.1.1. Classical (canonical) pathway

The canonical pathway is dependent on the activation of IKKβ and is triggered by proinflammatory cytokines such as TNF-α and IL-1 or by bacterial or viral infections (LPS or dsRNA respectively). The canonical or classical pathway to NF-κB activation implies the inducible phosphorylation and degradation of the inhibitory proteins IκBs. Upon stimulation, the IκB kinase complexe (IKKα/β/γ) is activated by phosphorylation of the activation loop found in IKKα and IKKβ and phosphorylates the inhibitory protein IκBα at serines 32 and 36. This phosphorylation event facilitates the interaction of IκBα with F-box/WD40 E3RS^{IκB}/β-TrCP, which allows ubiquitination of IκBα primarily at lysines 21 and 22 by an SKp1-Cullin-F-box (SCF)-type E3 ubiquitin ligase. This polyubiquitination event signals IκBα for degradation by the 26S proteasome. The nuclear localization signal of the NF-κB subunits is then freed from the IκB and thus the transcription factor can translocate to the nucleus and drive the expression of target genes (Figure 4) (60).

7.1.2. Non-canonical pathway

Recently an alternative pathway for NF-κB activation has been described (156, 187). This pathway is independent of the canonical IKK complex and was reported to be triggered only by specific members of the TNF family such as lymphotoxin β (LTβ) (37, 187), and B cell-activating factor belonging to the TNF family (BAFF also known as BlyS) (34), and CD40 (35) (figure 6). This non-canonical pathway involves the activation of the NF-κB inducing kinase (NIK), which activates IKK-α, which in turn phosphorylates the p100 precursor and targets it for ubiquitination. This polyubiquitination signal promotes the processing of p100 by the proteasome to remove the IκB-like C-terminal section and to generate the active form of NF-κB p52. This signaling pathway is mainly important for the expression of genes involved in the development, survival, proliferation and development of B lymphocytes.

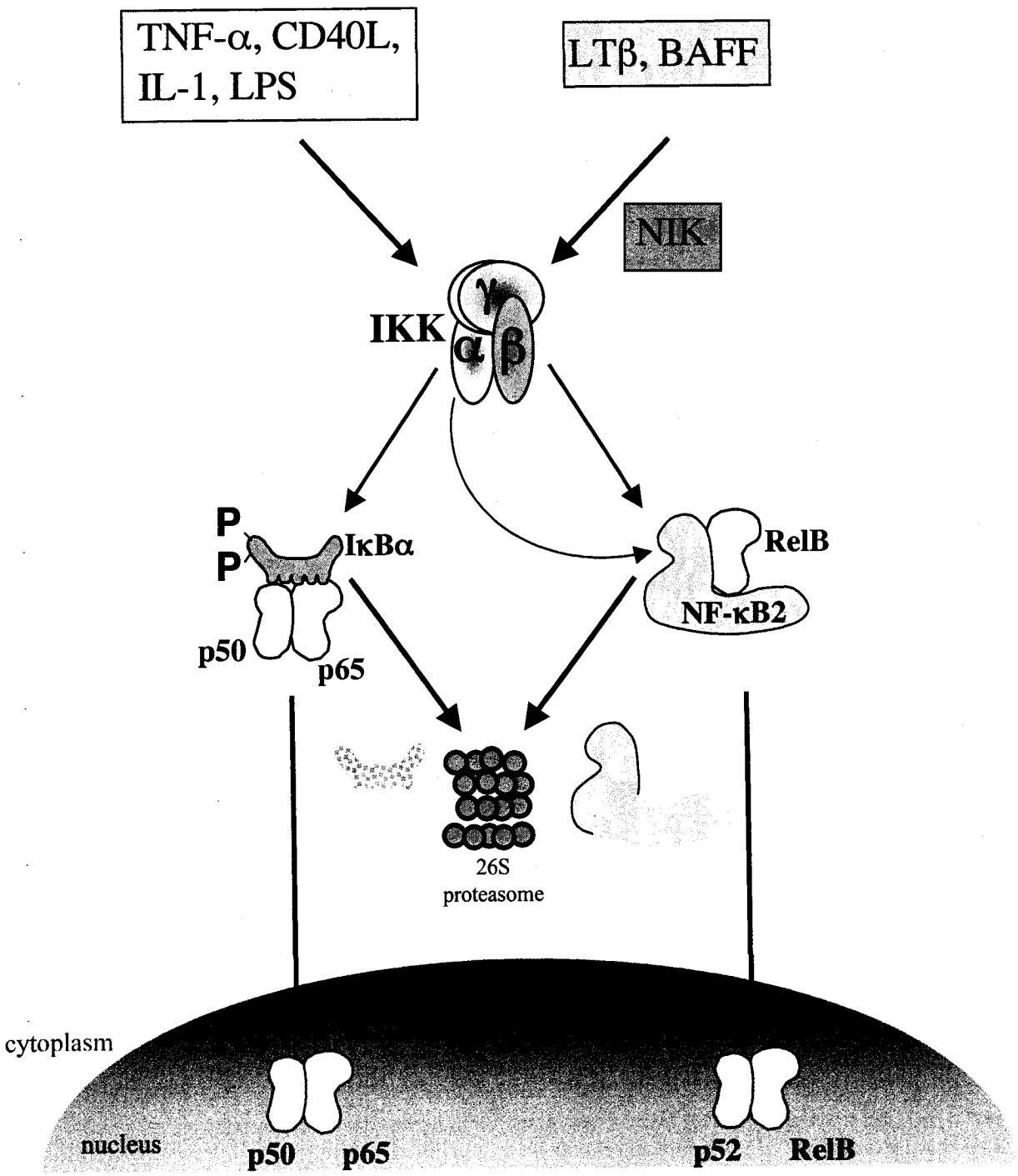
7.1.3. Other alternative pathways

Hypoxia or pervanadate treatment have been described to utilize atypical pathways leading to NF-κB activation, which result in a much slower and weaker activation as opposed to the response produced but the typical inducers TNF-α, IL-1 or LPS that activate the canonical pathway. Such weak stimulations lead to the phosphorylation of IκBα at Tyr-42 instead of Ser-32 and Ser-36. This pathway seems to be specific to IκBα, since tyrosine at position 42 is not conserved in the other members of the inhibitory proteins. Moreover, this phosphorylation does not promote the liberation of NF-κB dimers through IκBα degradation but rather leads to the dissociation of NF-κB through interaction with phosphoinositide-3 kinase (PI3K). The exact tyrosine kinases involved in this particular pathway are not known, although some members of the Src kinases family are suspected to be responsible for this Tyr-42 IκBα phosphorylation (14, 79).

Another particular stimulus that can lead to an alternative pathway for NF-κB activation is UV radiation, characterized this time by an unknown mechanism for IκBα

Figure 6. Classical and non-classical pathways leading to NF-κB activation

In figure 4 is described the classical pathway to NF-κB activation. Here, the classical and non-classical pathways are shown. Specific inducers such as LTβ or BAFF trigger NF-κB through the non-classical pathway, which implies processing of the precursor p100 (NF-κB2) into the mature p52 subunit. In this pathway, NIK is activated and induce the activation of the IKKα subunit, which in turn phosphorylate p100 (NF-κB2) and target it for ubiquitination. The ubiquitinated p100 is processed though the proteasome pathway where the IκB-like portion of p100 is removed to generate the active form of NF-κB p52. The p52/RelB dimer can thus translocate to the nucleus and promote expression of target genes.


TNF-α, CD40L,
IL-1, LPS
LTβ, BAFF
NIK
IKK
γ
α
β
P
P
IκBα
p50
p65
RelB
NF-κB2
26S
proteasome
cytoplasm
nucleus
p50
p65
p52
RelB

degradation. In this case, NF-κB is activated through degradation of IκBα by the 26S proteasome, but does not depend on Ser32 and Ser36 or Tyr42 phosphorylation (13, 102).

7.2. IKKε and TBK-1

Recently, two non-canonical IKK homologues - TBK-1 (NAK, T2K) and IKKε (IKK-i) - have been identified (18, 134, 140, 161, 174), and activation of either of these kinases results in NF-κB activation. IKKε was identified from databases as a homologue sequence to IKKα/β (134) while IKK-i was cloned from a subtractive hybridization screen as a novel LPS- inducible kinase related to IκB kinases (161). Unlike IKKα and IKKβ, which are constitutively expressed in most cell type, IKKε/IKK-i mRNA level is particularly high in spleen, in thymus and in peripheral blood lymphocytes (PBL) and is inducible in response to LPS and proinflammatory cytokines such as IL-1, IL-6, TNF-α (161). These observations suggest that this inducible IκB kinase might play a particular role in the immune response. Moreover, IKKε is inducible at the transcriptional level in other cell types following LPS, PMA, TNF-α or IL-1β stimulation (4, 93, 161). IKKε was shown to be present in a novel PMA-inducible IKK complex, distinct from the well-characterized IKKα/β/γ complex, that activates NF-κB following stimulation by PMA and T cell receptor activation, but not by TNF-α (134). This suggests a role for IKKε in NF-κB activation during T cell activation.

TBK-1 (TANK binding kinase-1), which is also known as NAK (NF-κB activating kinase) (174) or T2K (TRAF2 interacting protein kinase) (18), was identified by virtue of its association with TANK (TRAF family member-associated NF-κB activator) (140). TBK-1 is constitutively and ubiquitously expressed and as for IKKε, has a role in NF-κB activation.

IKKε and TBK-1 show sequence and structural homology to IKKα and IKKβ. Indeed, their catalytic domains exhibit 30% identity to IKKα/β and contains similar structures such as a N-terminal kinase domain, leucine-zipper and helix-loop-helix motifs within their C-terminal halves (figure 5) (161, 174). TBK-1 and IKKε have a molecular weight of 80 and 84 kDa respectively and show 64% homology in their overall amino

acid sequence. TBK-1 and IKKε share 67% identity in their kinase domain and 50% of their entire sequence.

IKKε and TBK-1 are not only similar in their amino-acid sequences, but also possess similar enzymatic activities. However, they have distinct functions to IKKα/β (78, 91). They are not part of the classical IKK complex, but both TBK-1 and IKKε phosphorylate IκBα on serine 36. However, as opposed to IKKα and IKKβ, they do not phosphorylate directly IκBα on serine 32. The physiological function of IKKε and TBK-1 in NF-κB activation remains to be defined. Beside this function, our group has recently shown that IKKε and TBK-1 mediate, through direct phosphorylation, the activation of IRF-3, a key transcription factor required for the interferon (IFN) gene induction in response to virus infection (159). For proper IFNβ transcription, both IRF3 and NF-κB activation are required, thus giving a double function to IKKε and TBK-1 in INFβ gene regulation and antiviral defense (figure 3) (reviewed in (71)). Thus, IKKε and TBK-1 have an important function in the antiviral defense and they have also been shown to be important for the proper IFNβ gene expression in response to LPS and dsRNA (69, 151).

7.2.1. Implication of IKKε and TBK-1 in NF-κB regulation

Until very recently, it was thought that all inducers of NF-κB converged to the activation of the classical IKKα/β/γ complex. However, another IKK complex which do not contain IKKα or β or γ was newly discovered (134). This complex, which is activated by certain inducers of NF-κB, such as PMA or TCR stimulation, can also phosphorylate serines 32 and 36 of IκBα, indicating that more that one IKK complex can be implicated in NF-κB activation. Although the exact composition of this IKK complex has yet to be elucidated, IKKε was identified as one of the component (134).

Similarly, over-expression of IKKε significantly induces NF-κB activation through the phosphorylation of both serines 32 and 36 of IκBα. However, IKKε phosphorylates only serine 36 of IκBα, suggesting that the new IKK complex would also include another serine 32/36 IκBα kinase (134, 161). Nevertheless, it is still unclear whether this IKKε-induced phosphorylation of both serines 32/36 of IκBα is a consequence of the activation of IKKα/β or another unidentified serines 32/36 IκBα

kinase. It was suggested that IKKε is capable of phosphorylating the activation loop of IKKβ, but the physiological relevance of this observation was not shown (135). Even though IKKβ is suspected to be activated by IKKε, studies from IKKε-/- MEFs suggest that it is unlikely for IKKε to be an IKKβ kinase; kinase activity of the classical IKK complex is similar in WT and IKKε-/- cells (93). There is also evidence that the activation of IKKβ/α is not the unique way to phosphorylate IκBα at serines 32/36. Experiments performed on Jurkat T cells treated either with PMA or activated by anti-CD3/CD28 antibodies showed that a S32/S36 kinase activity was immuno-precipitated with anti-IKKε antibody, but the S32 kinase activity was lost by washing with increasing amounts of urea. The remaining IKKε activity was directed to S36 only and no IKKα, IKKβ or IKKε was detected in the eluted fraction still capable of phosphorylating both serine residues (135). Thus, these results indicate that IKKε is involved in PMA/CD3 induction of NF-κB and requires the activation of another IκBα serine 32/36 kinase.

Similar to IKKε, TBK-1 phosphorylates IκBα and IκBβ in vitro, but only at serines 36 of IκBα and serine 23 of IκBβ as opposed to IKKβ, which phosphorylate both serines 32/36 of IκBα and 19/23 of IκBβ (174). Even if IKKε and TBK-1 do not appear to act as serines 32/36 IκB kinases, it is clear that they play a role in the activation of NF-κB (18, 134, 135, 161, 174), but the exact mechanism and their precise function is unclear. According to some reports, their implication appears to be dispensable for TNF-α, IL-1 or CD40 mediated NF-κB activation. Dominant negative form of IKKε blocks induction of NF-κB by PMA or TCR stimulation but not when stimulated by TNF-α or IL-1 (134). Similarly, a dominant negative form of TBK-1 was shown to block the effect of TANK on NF-κB activation, but had no effect on TNF-α, IL-1 or CD40 induced NF-κB activation (140). However, IKKε was shown to activate NF-κB through direct phosphorylation of TANK/I-TRAF and subsequent liberation of TRAF2 (129). This study suggested that IKKε is involved in TRAF2-mediated activation of NF-κB. TRAF2 is an adaptor protein involved in the signal transduction following TNF-α stimulation. Likewise, TBK-1, which is also called TRAF 2 interacting protein kinase (T2K), was implicated in NF-κB activation following TNF-α stimulation, through binding with

TRAF2. This would thus suggest that IKKε and TBK-1 are also implicated in TNF-α induced NF-κB activation.

Knockout mice studies also demonstrated a role for TBK-1 and IKKε in NF-κB activation. TBK1-/- embryos die from massive liver degeneration and apoptosis at day E14.5. This is very similar to the phenotype observed for p65-/-, IKKβ-/- and IKKγ-/- mice. As previously observed for p65-/-, IKKβ-/- and IKKγ-/- mice, the lethality of TBK1-/- mice could be rescued by crosslinking with TNFR-/- mice. Therefore TBK-1 is important for protection of TNF-α-induced cell death in liver cells, just as p65, IKKβ and IKKγ (18). According to other publications (174), TBK-1 contributes to NF-κB-dependent gene expression presumably by acting as an IKKβ kinase. Thus, if TBK-1 were truly an IKKβ kinase, its loss of function would impede IKKβ-mediated IκBα phosphorylation. However, in response to IL-1 or TNF-α, IκBα degradation and NF-κB binding occurs in TBK1-/- MEFs as in wild type MEFs, but no NF-κB transcriptional activity is observed. This suggests that TBK-1 is not required for IKKβ activity and IκBα degradation but is required for a subsequent step in NF-κB transcriptional activity (18). Mice lacking IKKε also showed an IκBα degradation pathway that is similar to the wild type mice, but the NF-κB-dependent gene transcription is impaired (93). TBK-1 and IKKε are thus suspected to regulate NF-κB activity independently of IκBα degradation, perhaps through phosphorylation of NF-κB subunits.

Recently, the identification of NAP1, a regulatory subunit of IKK-related kinases that have the potential to activate NF-κB, shed light on the role of TBK-1 in phosphorylating NF-κB subunits. Indeed, TBK-1-NAP1 complex was shown to phosphorylate p65 on serine 536 following TNF-α stimulation (52). IKKε was shown to also interact with NAP1, but direct phosphorylation of NF-κB by the IKKε /NAP1 complex has not been demonstrated. Recent studies have demonstrated that IKKε and TBK-1 in addition to IKKα, IKKβ and an unidentified kinase phosphorylated p65 on serine 536 constitutively or following IL-1 stimulation (22). The authors suggests that the phosphorylation of p65 on serine 536 promote the binding of p65 to the basal transcription machinery, in particular to TAFII31, and positively affect the transcription of the IL-8 gene.

7.2.2. Upstream signaling leading to IKKε and TBK-1 activation

As for IKKα and IKKβ, TBK-1 and IKKε are activated by phosphorylation of their activation loops. However, the MAPK kinase activation loop found in TBK-1 and IKKε contains only one serine residue ($EXXXS^{172}$) as opposed to the activation loops of IKKα/β which contains two ($S^{176}XXXS^{180}/S^{177}XXXS^{181}$). Moreover, in contrast to IKKα/β, MEKK-1 and NIK do not activate IKKε (135, 161). Until now, inducers such as viral infection, TLR3 stimulation by dsRNA, TLR4 stimulation by LPS, PMA, TCR stimulation by anti-CD3/CD28 antibodies are known to trigger IKKε and/or TBK-1 activation. However, the signaling pathways leading to their activation are not fully understood.

Recently, PAK1 (p21-activated kinase) and Rac1 were suggested to be upstream of IKKε and TBK-1 in the viral activation of IRF-3 (45). PAK1 is a serine-threonine kinase that is activated by binding to the Rho family small G proteins Rac and Cdc42. Rac and Cdc42 were already known to regulate NF-κB activity. We now know that NF-κB activation by active Ras, Raf-1 or Rac1 requires PAK1 activity. Indeed, it was demonstrated that PAK1 is required for NF-κB activity through multiple signaling pathways (51). One can thus propose that the activation of NF-κB through PAK1 might be dependent on IKKε and/or TBK-1. Moreover, PAK1 has the potential to induce p65 nuclear translocation, independently of IKKα or IKKβ. This suggest that IKKε and TBK-1-mediated activation of NF-κB might be independent of the classical IKKα/β, and IκBα phosphorylation on Ser32/36 and subsequent degradation. PAK1 can be activated by a number of stimuli including exposure of epithelial cells to IL1β and TCR stimulation in T cells (189) as well as by virus infection. (45) and LPS (9).

As mentioned above, NAP1 is now known to be a regulatory subunit of TBK-1 and IKKε, and is an upstream activator of these kinases in the TNF-α mediated NF-κB activation (52). More recently, the RNA helicase RIG-I (retinoic acid inducible gene I) was shown to have an essential function in dsRNA-induced innate antiviral responses through activation of NF-κB and IRF3. Thus suggesting that RIG-I is upstream of IKKε and TBK-1 (194).

8. Modulation of NF-κB activity by direct phosphorylation

Some NF-κB subunits, such as p105, p100, c-Rel and p65, are phosphoproteins that are constitutively phosphorylated in unstimulated cells. Several stimuli have the potential to increase this phosphorylation status, and modulate NF-κB activity (101, 124, 126). Inducible phosphorylation of p65 was observed following exposure of cells to hydrogen peroxide, TNF-α, PMA, IL-1 or LPS or anti-CD3/anti-CD28 antibodies (2, 15, 118, 148, 153, 181, 182).

Following IκBs degradation, nuclear translocation of NF-κB is not sufficient for proper NF-κB-dependent gene expression. An increasing amount of recent reports suggest direct phosphorylation of the NF-κB subunits as another level of regulation for NF-κB subsequent to IκBs degradation. The consequences of these phosphorylation events are variable and might modulate nuclear translocation of NF-κB, binding affinity to κB binding sites, association with coactivators or corespressors and/or effects on transactivation (reviewed in (152) and (31)). Inducible transcription factors such as activating protein 1 (AP-1) and cyclic AMP-responsive-element binding protein (CREB) are also regulated by phosphorylation of the DNA-binding subunits. Regulation of NF-κB through direct phosphorylation is a newly discovered level of regulation and the physiological relevance is not fully understood, but it probably provides a mechanism to fine tune NF-κB activity.

Seeing that p65 is the most frequently observed subunit of NF-κB, the majority of the reports focused their studies on this subunit. Although less is known on c-Rel regulation by direct phosphorylation, some reports indicate that c-Rel is also regulated by this process. Various kinases were shown to phosphorylate directly the NF-κB proteins, both in the cytoplasm and in the nucleus, as well as modulating its activity. Different sites of phosphorylation on the NF-κB proteins have been demonstrated and most of them are localized either in the RHD or in the transactivation domain (TD). Table 2 is a detailed review of the literature that has been published on the direct phosphorylation of NF-κB subunits and figure 7 is a schematic representation of p65 and c-Rel's main phosphorylation sites.

The effect of p65 phosphorylation seems to vary according to the target site. Indeed, when phosphorylation events occur in the RHD, such as on serine 276 or 311, p65 shows increased affinity to coactivators such as CBP/p300. Although it is still unclear if the phosphorylation of serine 536 in the TD increases the binding to p300, it seems that when phosphorylation events occur in the transactivation domain of p65 (serine 529 or 536), it could ease its interaction with other component of the basic transcriptional machinery such as TBP and TFIIB, thus regulating p65 transcriptional activity (16, 154, 188) (and reviewed in (31)). Several kinases are thus implicated in the regulation of p65 activity through direct phosphorylation and IKKs therefore have a double role in p65 activation, which is to phosphorylate IκBα and p65, thereby regulating NF-κB at two different levels.

Significant induced phosphorylation of c-Rel is triggered by PMA and CD28 as well as TNF-α treatment of T cells (20, 116). Even though less information is published on c-Rel direct phosphorylation, some reports indicate that the transactivation domain is a target for regulatory phosphorylation. Indeed, TNF-α-induced activation of c-Rel requires the phosphorylation of serine 471 in its transactivation domain. Although the specific kinase has yet to be identified, PI3K and PKCζ are though to be implicated in this process (116, 117). Serine 451 in mouse c-Rel is also phosphorylated in the transactivation domain of c-Rel but the regulatory effect remains to be elucidated (46).

Figure 7. Schematic representation of p65 and c-Rel target amino acids for direct phosphorylation

An exhaustive review of the known kinases and target sites for p65 and c-Rel direct phosphorylation is represented in table 2. Here is a summary of all known kinases and phosphorylation sites located either in the RHD or the transactivation domain. The RHD of p65 is targeted for phosphorylation on serine 276 by PKAc and MSK1, and serine 311 by PKCζ. To date, no phosphorylation sites in the RHD of c-Rel have been described. Phosphorylation of the RHD of p65 is believed to increase interactions with coactivators. Serine 529 of p65 transactivation domain is targeted by CKII and by the TAX-activated IKK complex. Serine 536, which is the most studied phosphorylation site of p65 transactivation domain, is phosphorylated by IKKα, IKKβ, IKKε, TBK-1 and RSK1. The phosphorylation of p65 in its transactivation domain leads to increased activity in gene transactivation. Less is known for the kinases and phosphorylation sites in c-Rel transactivation domain. However, serine residue 471 was shown to be essential for TNF-α-induced c-Rel transactivation. The direct kinase is not known, but PKCζ and PI3K are believed to participate in this event. An unknown kinase, TD kinase, was shown to bind to and phosphorylate c-Rel in its transactivation domain, however the effect of this phosphorylation has not been investigated. Another group of kinases such as GSK3β, NIK, PI3K/AKT are suspected to act as NF-κB kinases based on knockout studies

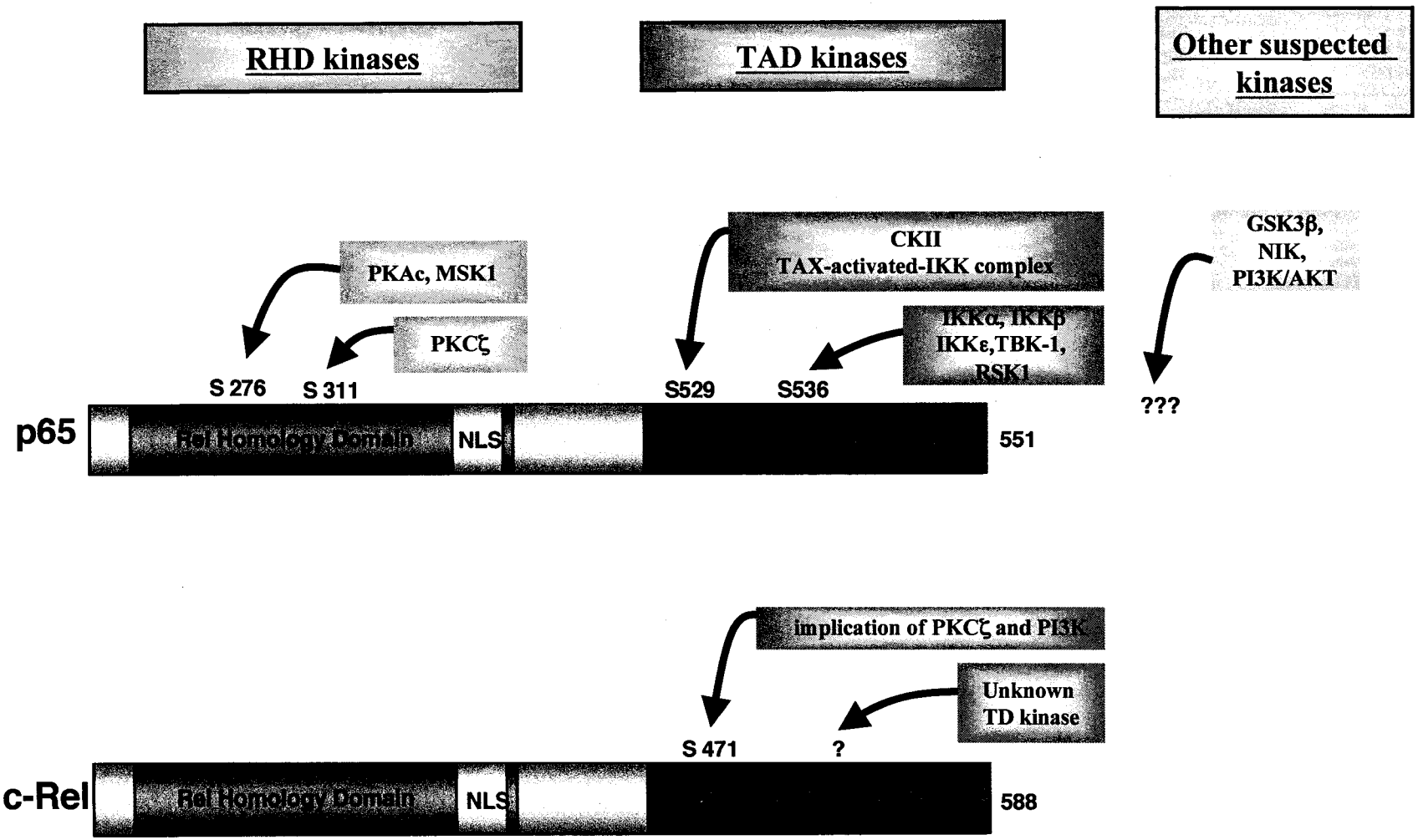

RHD kinases
TAD kinases
Other suspected kinases
PKAc, MSK1
PKCζ
CKII
TAX-activated-IKK complex
IKKα, IKKβ
IKKε,TBK-1,
RSK1
GSK3β,
NIK,
PI3K/AKT
S 276
S 311
S529
S536
???
p65
Rel Homology Domain
NLS
551
implication of PKCζ and PI3K
Unknown
TD kinase
S 471
?
c-Rel
Rel Homology Domain
NLS
588

Table 2. Direct phosphorylation of NF-κB subunits

NF-κB	a.a. position / domain	Involved kinases	Inducer	Effect of the phosphorylation	References
p65	Ser 276 (RHD)	PKAc	LPS	· Enhance NF-κB transcriptional activity · Increase interaction with coactivator CBP/p300 · Phosphorylated p65 complex is more effective in displacing transcriptionally repressive histone deacethylase (HDAC) complexes, which are often bound to κB enhancers of target genes in unstimulated cells. · Phosphorylation occurs in the cytoplasm.	(197, 198)
p65	Ser 276 (RHD)	MSK1	TNF-α	· Enhance NF-κB transcriptional activity · Increase interaction with coactivator CBP/p300 · Phosphorylation occurs in the nucleus	(178)
p65	Ser 311 (RHD)	PKCζ	TNF-α	· Disruption of the PKCζ gene lead to impaired NF-κB activity · Enhance transacriptional activity · Increase interaction with coactivator CBP/p300	(43, 99)
p65	Multiple sites in the RHD	PKCζ and p21 RAS	PKCζ- and RAS-derived signals like TNF-α	· In endotheliaql cells, p65 is inducily phosphorylated in the RHD by PKCζ- and RAS-derived signals. · Dominant negative forms of p21 ras or PKCζ inhibit the transcriptional activity of p65 without interfering with DNA binding.	(2)
p65	Ser 529 (TD)	Not identified (probably CKII)	TNF-α	· Increased p65 transcriptional activity · No effect on nuclear translocation · No effect on DNA binding	(181)
p65	Ser 529 (TD)	CKII	TNF-α or IL-1	· Activation of NF-κB	(15, 182)
p65	Ser 536 (TD)	IKK α/β	TNF-α	· *In vivo*, p65 is phosphorylated after TNF-α stimulation at the same time that IκBα is phosphorylated · Cytoplasmic extract of TNF-α stimulated cells phosphorylate serine 536 *in vitro*. · Endogenous IKK complex, overexpressed IKKα and β and recombinant IKKβ phosphorylate serine 536 of p65 *in vitro*. · TNF-α- induced IKK phosphorylation of p65 on serine 536 is mediated through TRAF2, TRAF5 and TAK1 signaling pathway	(148, 149)
p65	Ser 536 (TD)	IKK α and IKKβ	LPS	· LPS induces p65 phosphorylation on serine 536 in monocytes/macrophages · IKKβ plays an essential role in LPS-induced p65 phosphorylation on serine 536 and IKKα is partially required. · LPS-induced phosphorylation of p65 on serine 536 increase its transcriptional activity.	(193)
p65	Not identified	Purified recombinant IKKα/β	Not applicable	· Not shown if the phosphorylation is physiologically relevant.	(120)
p65	Not identified	Purified IKKα/β/γ complex from mammalian cells	Cytokine	· In vitro phosphorylation · Stringent washing dissociates the kinase activity from IKK · Not shown if the phosphorylation is physiologically relevant.	(121)
p65	Ser 536 (TD)	IKKα	HTLV-1 Tax oncoprotein	· Activation of NF-κB	(130)
p65	Ser 536 (TD)	IKKα and NIK	LTβR stimulation	· Serine 536 is phosphorylated following LTβR stimulation and this phosphorylation is inhibited by the kinase-dead dominant-negative mutant of NIK or IKKα	(84)
p65	Ser 536 (TD)	RSK-1	Activation of RSK1 by tumor suppressor p53	· Phosphorylated p65 has lower affinity for IκBα	(17)

p65	Ser 536 (TD)	TBK-1 (a.k.a. NAK or T2K)	TNF-α	· Cells lacking TBK1 show less NF-κB activity · After TNF-α or IL-1 stimulation in TBK-1 -/- cells, IκBα degradation and subsequent nuclear translocation of p65 occur normally. · TBK-1 phosphorylates p65 but the effect has not been tested. However it was suggested that it could activate p65 transcriptional activity.	(18, 52)
p65	Ser 536 (TD)	IKKα, IKKβ, IKKε, TBK-1 and an unknown kinase	IL-1	· Constitutive and inducible IL-1 phosphorylation of p65 on serine 536 is mediated by at least these five kinases · Ser536 phosphorylation of p65 is independent of JNK, p38, ERK and PI3K pathway but is induced by proteasome inhibition through the IKK complex · Ser536 phosphorylated p65 is recruited to IL-8 promoter · Ser536 is contained in a FXXoo motif of p65, which couple p65 to the basal transcription machinery, TAFII31 particularly	(22)
p65	Ser 536 (TD)	IKKβ	Anti-CD3/CD28 TCR stimulation	· T cell costimulation induces p65 phosphorylation on serine 536, which require IκBα phosphorylation and IKKβ activation · T-cell costimulation-induced p65 phosphorylation on ser536 occurs in the cytoplasmic NF-κB:IκB complex · Phosphorylated p65 stays longer in the cytoplasm following TNF-α stimulation of MEFs as compared to p65 S536A mutant · Kinetic of p65 ser536 phosphorylation paralleled IκBα and IKK phosphorylation · This pathway leading to p65 ser536 phosphorylation is regulated by Cot (Tp12), RIP, PKCq, NIK but independent of PI3K/Akt pathway ·	(118)
p65	Not identified	GSK3β	TNF-α	· Cells lacking GSK3β show less NF-κB activity · GSK3β phosphorylates p65 *in vitro* · After stimulation of GSK3β-/- cells, IκBα degradation and subsequent nuclear translocation of p65 occur normally	(72, 155)
p65	Not identified	Akt/PI3K	IL-1	· PI3K inhibitors reduce NF-κB activity · Not known if it is a direct phosphorylation · Most likely to activate IKKβ wich phosphorylated p65	(115, 163)
p65	Serine 535	recombinant CaMKIV	n/a	· Recombinant CaMKIV phosphorylate p65 on serine 535 in vitro · Mutation of srine 535 into alanine abolish CaMKIV induced transcriptional activity as well as uninduced p65 activity. · S535A mutation blocks the recruitment of CBP and the release of SMRT induced by CaMKIV	(5, 83)
Dorsal	Ser317			· Signal-induced phosphorylation occurs on Dorsal while still bound to Cactus. · Required for signal-induced nuclear translocation. · Not required for basal nuclear translocation · Not required for signal-induced Cactus degradation · Ser317 is completely conserved among Rel/NF-κB members and it was suggested that this phosphorylation might be a general mechanism for regulating signal-induced NF-κB nuclear translocation.	(42)
c-Rel	Serine 451 of mouse c-Rel (TD)	Mammalian 66 kDa TD kinase suggested to be ERK1	Not identified	· A cytosolic mammalian 66 kDa kinase binds murine c-Rel both *in vitro* and *in vivo*. · Phosphorylate c-Rel in the transactivation domain to an ERK consensus site between a.a 447-455. · Recombinant ERK1 phosphorylates murine c-Rel in the TD at serine 451 *in vitro*.	(46)
c-Rel	Ser 471 (TD)	Not identified	TNF-α	. Mutation of Ser 471 in the human c-Rel TAD abrogate TNF-α-induced NF-κB activity in Jurkat T cells. . PI3K and PKCζ are participating in TNF-α-induced c-Rel phosphorylation but have not been shown to directly phosphorylate c-Rel.	(116, 117)
c-Rel	Not identified	Not identified	PMA and CD28	· PMA and CD28 stimulation of T cells increase c-Rel phosphorylation	(20)

Unless mentioned, studies were with human c-Rel and human p65

9. Termination of NF-κB-mediated gene transcription

NF-κB transcription factors are ideal for the expression of genes involved in the innate immune response as they are rapidly activated and do not require de novo protein synthesis. This provides the cell with a very rapid way to produce cytokines and other potentially toxic proteins to clear out various pathogens. As those NF-κB-dependent newly synthesized proteins might harm the cell itself, the NF-κB pathway needs to be tightly regulated to end the expression of such genes when it is no longer required.

Termination and down-regulation of NF-κB activity involves a variety of mechanisms that target several levels of the activation pathway. For example, IKKα and β can be the target of their own kinase activity. Heavy auto-phosphorylation of IKKα and IKKβ is thought to decrease their kinase activity, thus preventing or delaying NF-κB activity (38). Transcriptional repressors such as Twist 1/2 (164) or replacement of the active NF-κB dimer by an inactive dimer (146) was recently shown as a mechanism utilized by the cell to end NF-κB driven transcription.

Until now, the main mechanism known to terminate NF-κB activation is achieved through a negative feedback loop. As mentioned previously, the NF-κB transcription factors are able to promote the expression of the inhibitory protein IκBα (or others IκBs). The IκBα protein harbors both NLS and NES in its amino acid sequence (figure 1) and thus enables it to shuttle between cytoplasm and nucleus. The newly synthesized IκBα can enter into the nucleus and take up the NF-κB proteins to bring them back to the cytoplasm in their inactive form. However, this mechanism of negative feedback occurs rapidly and is believed to terminate the early NF-κB activation following various stimuli.

Recently, evidence for another system that might be involved in the termination of p65 response was observed for the first time in mammals (145). This new mechanism is a proteasome-dependent pathway of p65 response termination. The authors of this report stated that after activation, p65 is polyubiquitinated and then degraded by the proteasome in a DNA-binding manner into the nucleus. This has been observed in the absence and in presence of IκBα, indicating that both IκBα-dependent and –independent mechanisms for p65 activity termination occur synergistically. Termination of Rel proteins by proteasome-mediated degradation has also been detected in *Drosophila* (89).

10. Specific aim of this research project

Our group has recently demonstrated that the two IKK homologues -IKKε and TBK-1- can trigger antiviral immunity through direct phosphorylation of the IRF3/IRF7 transcription factors, which are key regulators of the interferon response (159). Given that IKKε can regulate transcription factor activity and that as c-Rel, its expression is restricted to hematopoietic cells, we hypothesized that IKKε may modulate c-Rel activity by direct phosphorylation of its TAD. Since TBK-1 is very similar to IKKε and that most of the substrates for those kinases are the same, in a second order, we also studied the relation between c-Rel and TBK-1. The aim of this research project is thus to demonstrate first that c-Rel is a substrate for IKKε/TBK-1 and then characterize the phosphorylation sites and study the effect of this phosphorylation on c-Rel behavior. After analysis of the amino acid sequence of c-Rel, we found three potential consensus sites for IKKε/TBK-1. The strategy is to find which of these sites is targeted and more specifically which serine residue is the target for IKKε/TBK-1 phosphorylation. Because NF-κB activation begins with nuclear translocation followed by binding to DNA and then activation of gene transcription, each of these steps will be studied in relation to c-Rel phosphorylation by IKKε/TBK-1.

CHAPTER II

MATERIAL AND METHODS

1. Cell culture and plasmids

293T and HeLa cells were grown in DMEM supplemented with 10% calf serum, Jurkat T cells were grown in RPMI with 10% fetal bovine serum and incubated at 37°C with 5% CO_2. For the pcDNA3.1 zeo IKKε wild type construct, the coding sequence of human IKKε gene (bp 327-2477) were amplified by PCR from an HTLV-1 infected T lymphocyte cDNA library (Clonetech) and cloned (*EcorR1/BglII*) into the flag- or myc-tagged pCDNA3.1 zeo expression vector. The human TBK-1 cDNA was obtained from Origene Technology and amplified by PCR for subcloning (*Not1/Xba1*) in the flag- or myc-tagged pCDNA3.3 zeo expression vector. The kinase dead variant of IKKε which have been previously described to act as a dominant negative mutant (134, 161) and of TBK-1 were generated by site-directed mutagenesis substituting lysine 38 for an alanine residue (K38A) into the kinase domains of the proteins. Plasmids encoding myc-IKKα, flag-IKKβWT (108), flag-IKKβDN, IκBαWT, IκBα2N, (10) have been described previously.

GST-c-RelTD (aa 422-588), GST-c-Rel A (aa 422-472), GST-c-Rel B (aa 473-522), GST-c-Rel C (aa 523-588) constructs were generated by PCR amplification of the pCMVBL-c-Rel and subcloned with *BamH1/EcoRI* in the pGEX4T2 vector (Amersham). The myc-tagged c-Rel was obtained by cloning into the myc-tagged pCDNA3.1 zeo vector with *EcoRI/HindIII* of the PCR-amplified c-Rel from pCMVBL-c-Rel construct (forward primer: 5'-ATA TAA GCT TAG CGG AGC CAT GGC CTC CGG TGC GTA TAA-3', Reverse primer: 5'-ATC GGA ATT CTA CAA AAT GCT GCA TCT ATA T-3'). The pGL3-IRF4 CD28RE (also named 0.4-kbIFR4-PRO-pGL3) had been generated by digestion of the 1.2-kbIFR4-PRO-pGL3 with *NotI/PstI* as described previously (158).

2. Mutagenesis

All serine to alanine mutations of the various c-Rel constructs were generated by PCR amplification of the myc-tagged c-Rel plasmid with mutated primers using the Quick-change mutagenesis kit from Stratagene. The following primers were used to produce GST-c-RelTD S443A and GST-c-Rel peptide A S443A forward primer: 5'-GTC GGA ATG GAA GCG GCA TCC ATG CCA TCA GC-3', reverse primer: 5'-GCT GAT GGC ATG GAT GCC GCT TCC ATT CCG AC-3'; GST-c-RelTD S447A and GST-c-

Rel peptide A S447A forward primer: 5'-GGA AGC GTC ATC CAT GCC AGC AGC AGA TTT ATA TGG-3', reverse primer: 5'-CCA TAT AAA TCT GCT GCT GGC ATG GAT GAC GCT TCC-3'; GST-c-RelTD S509A and GST-c-Rel peptide B S509A forward primer: 5'-CCA TCA GAT GTC CGC TTC CAG TAT GTC AGC AGG C-3', reverse primer: 5'-GCC TGC TGA CAT ACT GGA AGC GGA CAT CTG ATG G-3'; GST-c-RelTD S513A and GST-c-Rel peptide B S513A forward primer: 5'-CCT CTT CCA GTA TGG CAG CAG GCG CCA ATT CC-3', reverse primer: 5'-GGA ATT GGC GCC TGC TGC CAT ACT GGA AGA GG-3'; GST-c-RelTD S566A and GST-c-Rel peptide C S566A forward primer: 5'-GGT TTT GTT CAA GAT AGT CAG TAT GCA GGT ATT GGC AGT ATG C-3', reverse primer: 5'-GCA TAC TGC CAA TAC CTG CAT ACT GAC TAT CTT GAA CAA AAC C-3'; GST-c-RelTD S570A and GST-c-Rel peptide C S570A forward primer: 5'-GTC AGT ATT CAG GTA TTG GCG CTA TGC AAA ATG AGC AAT TGA GTG-3', reverse primer: 5'-CAC TCA ATT GCT CAT TTT GCA TAG CGC CAA TAC CTG AAT ACT GAC-3'.

The double mutation of the myc-c-Rel construct has been generated with the following primers: myc-c-Rel mutant A (S443/447A) forward primer: 5'-GTC GGA ATG GAA GCG GCA TCC ATG CCA GCA GCA GAT TTA TAT GG-3', reverse primer: 5'-CCA TAT AAA TCT GCT GCT GGC ATG GAT GCC GCT TCC ATT CCG AC-3; myc-c-Rel mutant B (S443/447A) forward primer: 5'-CCA TCA GAT GTC CGC TTC CAG TAT GGC AGC AGG CGC C-3', reverse primer: 5'-GGC GCC TGC TGC CAT ACT GGA AGC GGA CAT CTG ATG G-3' (no positive clone were obtained from this mutagenesis); myc-c-Rel mutant C (S443/447A) forward primer: 5'-GTC AGT ATG CAG GTA TTG GCG CTA TGC AAA ATG AGC AAT TGA GTG-3', reverse primer: 5'-CAC TCA ATT GCT CAT TTT GCA TAG CGC CAA TAC CTG CAT ACT GAC-3.

3. Immunoblot analysis and antibodies

Cells were washed once in PBS and lysed in 50 mM Tris-HCl pH 7.4, 1% NP40, 150 mM NaCl, 5 mM EDTA, 10% glycerol supplemented with 5 μg/ml aprotinin,

5 μg/ml leupeptin, 5 μg/ml pepstatin, 1 mM sodium orthovanadate, 40 mM β-glycerophosphate, 10 mM *p*-nitrophenylphosphate and 30 mM NaF (lysis buffer) for 15 minutes on ice, followed by three freeze/thaw rounds. After centrifugation at 13000 rpm for 20 minutes at 4°C, the supernatant (whole cell lysate) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membrane (Bio-Rad). The membrane was blocked in PBS containing 0.05% Tween-20 and 5% nonfat dry milk for 1 h and incubated with primary antibody, anti-c-Rel (1:1000) (19) or anti-c-Rel sc-70 from Santa Cruz (1μg/ml), anti-IκBα MAD3-10B (1:1000), anti-IκBα phosphoserine 32 (1:1000; Cell signaling), anti-α-actin (Chemicon), anti-flag (1μg/ml; Sigma), anti-myc (1μg/ml; Sigma) in blocking solution. After five 5 minutes washes in PBS containing 0.05% Tween 20, the membranes were incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG (1:2000-1:10000; KPL laboratories) in blocking solution. Immunoreactive proteins were visualized by enhanced chemiluminescence (Perkin Elmer).

4. Metabolic labeling.

Twenty-one hours post-transfection, 293T cells were washed twice in phosphate-free DMEM medium (MP Biomedicals) and further cultured in phosphate-free DMEM medium supplemented with 1 mM glutamine, 10% dialyzed FBS and 0.2 mCi/ml [^{32}Pi] orthophosphoric acid (^{32}P). After 3 hours, cells were washed in cold PBS, lysed in lysis buffer and subjected to immunoprecipitation using protein G sepharose beads precoupled with anti-myc antibodies (1 μg). Immunocomplexes were resolved by SDS-PAGE electrophoresis and transferred onto nitrocellulose membrane. Incorporation of ^{32}P was revealed by exposing the membrane to autoradiography. Immunoprecipitation of myc-flagged-c-Rel was verified by immunoblot analysis using anti-c-Rel antibodies as described above.

5. *In vitro* kinase assay.

Recombinant GST-fusion proteins used as substrates were produced in *E. coli* BL21 bacteria transformed with appropriate plasmid constructs (pGEX-4T2) encoding for GST-fusion protein. A single colony from each transformation was grown in 2ml of

LB (Luria Broth) overnight. The culture was then transferred to a larger volume of LB (1:100 dilution) and grown until OD at 600nm reach 0.6. The production of the recombinant protein was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3h at 37°C. After induction, bacterial pellet was lysed in PBS with 1% Triton-X-100 and sonicated for 6 minutes by pulses of 10 seconds ON, 10 seconds OFF at 30% efficiency (3 minutes of total sonication). Bacterial lysate was cleared by centrifugation for 15 minutes at 10 000 rpm at 4°C. GST-peptides were then purified from the cleared bacterial lysate by pull-down with glutathione sepharose beads for 30 minutes at room temperature followed by 3 washed with 0.1% Triton X-100 PBS and eluted with elution buffer (150 mM NaCl, 50 mM Tris pH7.5, 20 mM glutathione). Recombinant kinases were produced according to the protocol from the manufacturer (Promega) by expression of appropriate pcDNA3.1 zeo encoding construct in an *in vitro* transcription/translation system made from rabbit reticulocytes lysate. Immunoprecipitations for the *in vitro* kinase assay were performed by incubating rabbit reticulocytes lysates for 4 h at 4°C with protein G-Sepharose beads precoupled to anti-myc or anti-flag antibodies. Immunocomplexes were washed twice in 50 mM HEPES pH 7.4, 2 mM EDTA, 150 mM NaCl, 10 % glycerol, 1% NP 40, 0.1 mM sodium orthovanadate, 20 mM β-glycerophosphate, 10 mM p-nitrophenylphosphate, 5 mM NaF, 5 μg/ml aprotinin and 5 μg/ml leupeptin and twice in IKK kinase buffer (20 mM HEPES, 150 mM NaCl, 20 mM $MgCl_2$, 1 mM DTT, 0.1 mM sodium orthovanadate, 20 mM -glycerophosphate and 10 mM p-nitrophenylphosphate) and used in the kinase reaction. The kinase reaction was performed by incubation of immunocomplexes with 10 μCi γ-^{32}P-ATP, 20 μM ATP, 1.0-3.0 μg GST substrate at 30°C for 30 min in kinase buffer. Proteins were resolved by 10% acrylamide SDS-PAGE electrophoresis. The gel was stained with Coomassie blue for 15 min, destained in 10% ethanol-10% acetic acid, dried and exposed to Biomax XR film (Kodak) (See figure 8 for schematic of the method).

6. Immunofluorescence

293T cells were plated onto coverslips and transfected the next day using calcium phosphate. After 24h of expression, the coverslips were collected and washed once in cold PBS. Cells on coverslips were then fixed with a mixture of methanol and acetone

(ratio 1:1) for one minute at room temperature. Coverslips were then washed 3 times with cold PBS. The staining was performed for 1h at room temperature in the dark with a mixture of Cy3-labeled anti-myc (1:1000) and FITC-labeled anti-flag (20μg/ml) in PBS. Cells were then washed twice with PBS and then incubated with Hoescht nuclei stain for 2 minutes. Cells on coverslips were washed three more times with PBS followed by a single wash in distilled water. Coverslips were mounted on microscope slides with anti-fade mounting media (company). Slides were analyzed with an Olympus fluorescent microscope and pictures were taken and analyzed with the ImagePro Plus software from Media Cybernetics.

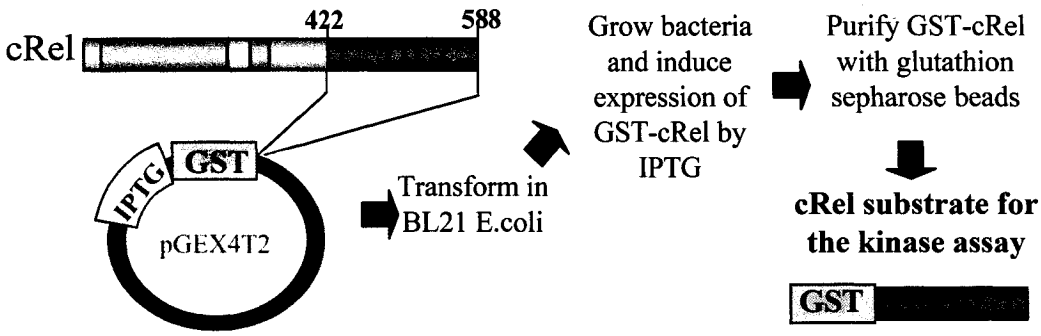
7. Reporter gene assays

293T cells were plated in 24 well-plates the night before transfection. Cells were then transfected using the calcium phosphate method with constant amounts of pGL3-IRF4 CD28RE plasmids as reporter gene, pRLTK as internal reference, c-Rel expressing plasmid and an increasing amount of IKKεWT expressing plasmid. The total amount of transfected DNA was adjusted with empty pcDNA3.1 vector. After 24h of expression, cells were harvested and lysed in 100μl of passive lysis buffer (Promega) and left for 15 minutes agitating to permit efficient lysis. Then, 10μl of cell lysate was used to measure the luciferase and renilla signal using reagents from the dual luciferase kit from Promega. Ratios of the luciferase signal on the renilla signal were calculated and the results are expressed in fold induction of the ratio value compared to the sample transfected with the reporter gene alone (pGL3-IRF4 CD28RE and pRLTK).

Figure 8. Schematic representation of the *in vitro* kinase assay procedure

To perform *in vitro* kinase assay, recombinant GST-c-Rel TD fusion protein was produced using a pGEX4T2 vector expressing the transactivation domain of c-Rel. The vector was transformed into BL21 E. coli bacteria and induced by IPTG. The GST-c-Rel TD fusion protein was purified with sepharose beads and used as substrate for the kinase assay. The kinases used were generated by the *in vitro* transcription/translation of the corresponding tagged expressing plasmid in rabbit the reticulocyte system from Promega. Kinases were purified by immunoprecipitation and the kinase reaction was performed on the washed beads from the immunoprecipitation. The reaction mix, containing the GST-c-Rel TD substrate, the immunopurified kinas and ^{32}P labeled ATP and the kinase buffer was left for 30 minutes at 30C. This step will add ^{32}P to the residues targeted by the kinase using the ^{32}P labeled ATP. The reaction was stopped by addition of SDS loading dye and samples were run on SDS-PAGE, stained with Coomassie Blue. Gels were dried and exposed to autoradiography. In conditions were the substrate is phosphorylated, the ^{32}P signal is seen on the autoradiography. By Coomassie Blue the band corresponding to the substrate is visible whether it is phosphorylated or not.

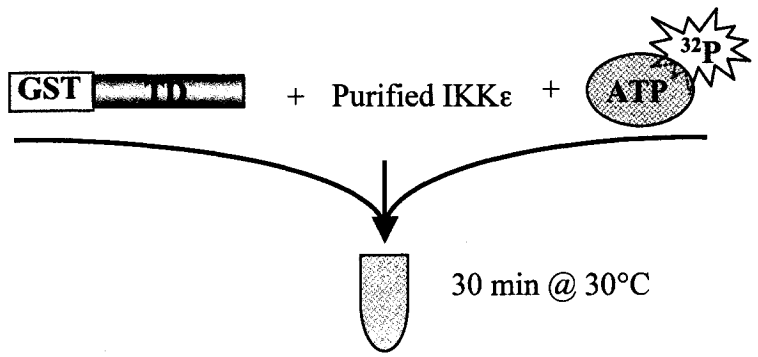
1. Produce recombinant GST-cRel TD fusion protein



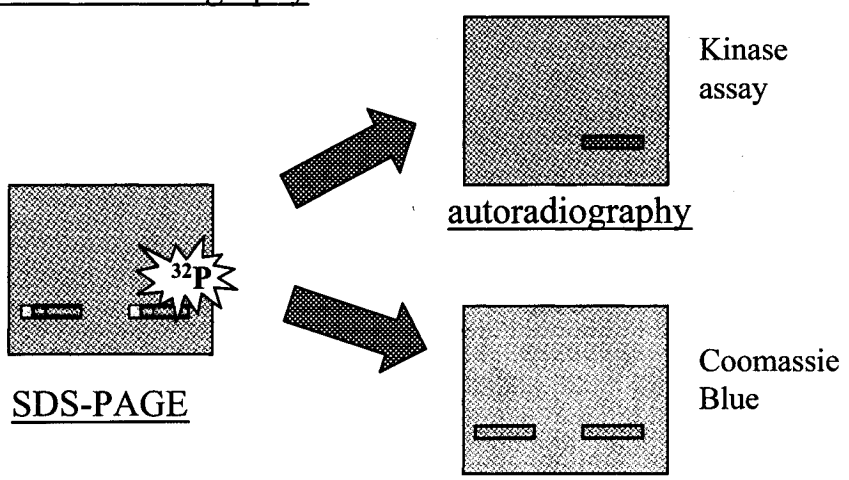
2. Produce recombinant kinases

3. Purify kinases by immunoprecipitation

4. Perform *in vitro* kinase assay



5. Run reaction mix on SDS-PAGE, stain gel with Coomassie Blue, dry and expose to autoradiography



CHAPTER III

RESULTS

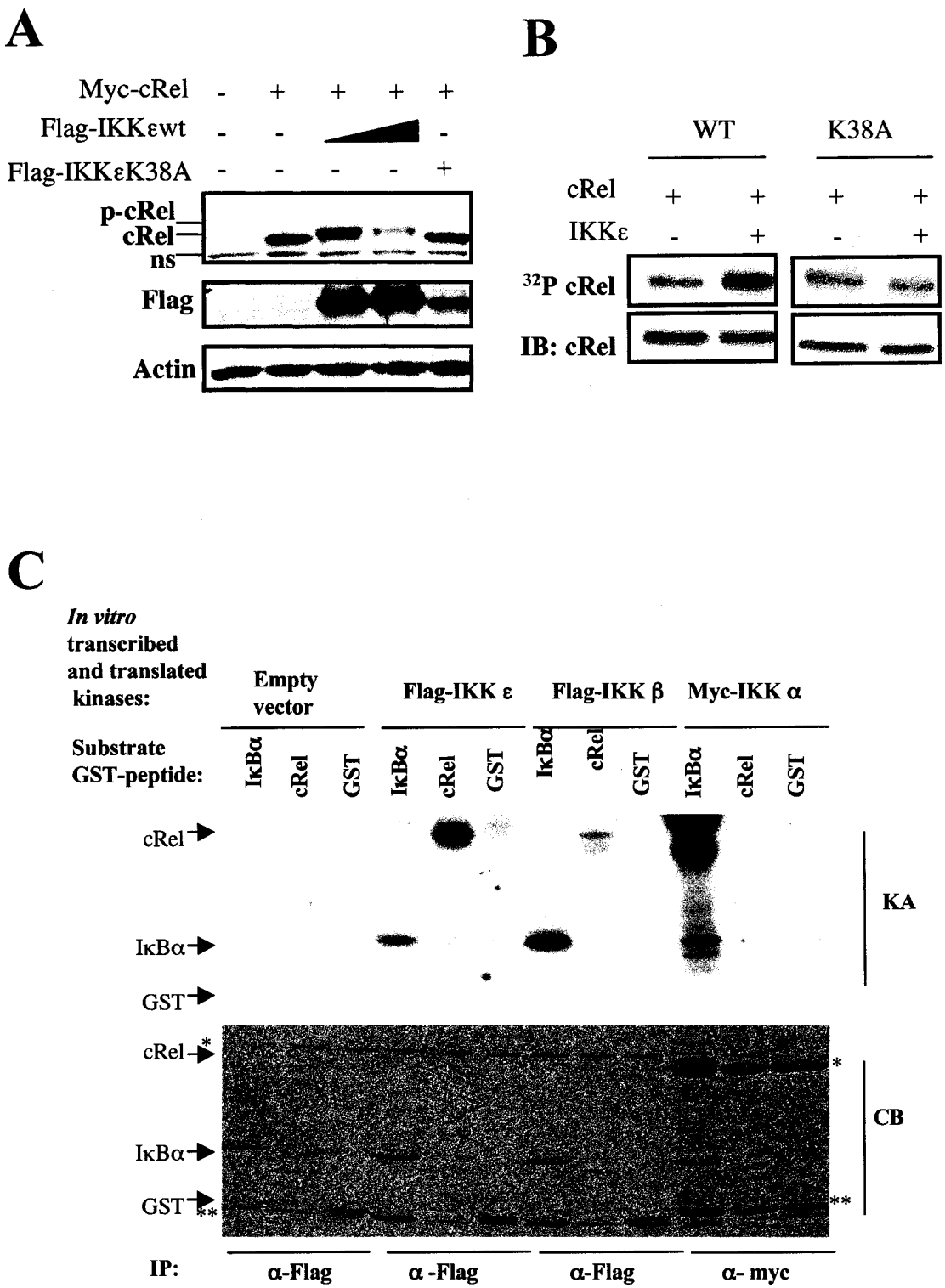
1. c-Rel is phosphorylated *in vivo* by IKKε and TBK-1.

IKKβ is known to phosphorylate the NF-κB p65 subunit thereby enhancing p65 transcriptional activity (148, 149). Moreover, very recently IKKβ, IKKα, IKKε and TBK-1 and another unknown kinase were shown to induce constitutive and IL-1-induced p65 phosphorylation on serine 536 (22). Given that c-Rel is specifically expressed in lymphoid cells, and that IKKε is the only IKK kinase to be restricted to those cells, we asked whether this kinase could phosphorylate c-Rel in order to regulate its activity. Due to the high homology between IKKε and TBK-1, and until now were shown to have the same substrates, we also tested whether TBK-1 could phosphorylate c-Rel. Both IKKε and TBK-1 are constitutively active upon transfection and the single mutation of lysine 38 into alanine is sufficient to abolish their kinase activity (161). In order to verify whether c-Rel is a substrate for IKKε and/or TBK-1, HeLa cells were cotransfected with plasmids expressing myc-tagged c-Rel and flag-tagged-IKKε wild type (WT), -IKKε K38A or -TBK-1 WT. After 24h of expression, cells were harvested and whole cell lysates were analyzed by immunoblotting using anti-c-Rel, anti-flag or anti-actin antibodies. As shown in figure 9, c-Rel migrates more slowly in the presence of IKKε WT (panel A) but not when it is coexpressed with IKKε K38A. Similar results were observed in 293T cells (data not shown). Thus, the slower migration pattern is dependent on the kinase activity of IKKε and TBK-1.

In order to confirm that this shift in the migration pattern is associated with phosphorylation, *in vivo* metabolic labeling with ^{32}P was performed in 293T cells cotransfected with plasmids encoding myc-tagged c-Rel and flag-tagged IKKε WT or K38A. As observed in panel B of figure 9, c-Rel is phosphorylated at the basal level, but the phosphorylation signal is increased with the expression of IKK-ε WT and not when coexpressed with the kinase dead variant. Taken together, these results strongly suggest that c-Rel is phosphorylated *in vivo* in response to IKKε or TBK-1 expression. The slower migration of phosphorylated cRel in figure 9, panel B is not observed. This might be explained by the smaller migration path of the mini gel used in SDS-PAGE for the protein separation of the *in vivo* metabolic labeling assay, as compared with a bigger gel in western blotting of panel A. To further confirm the phosphorylation of cRel, we have

Figure 9. IKKε induces *in vivo* and *in vivo* cRel phosphorylation.

A) Hela cells were cotransfected with plasmids encoding myc-tagged cRel (2 μg) and flag-tagged IKKεwt (1 or 2 μg) or flag-tagged IKKεK38A (1 μg) or empty vector. Whole cell extracts were resolved by SDS-PAGE and subjected to immunoblot analysis using anti-cRel, anti-flag or anti-actin antibodies. Ns: non specific band. **B)** HEK293T cells were cotransfected with myc-tagged cRel (2 μg) and flag-tagged IKKεwt (500ng) or flag-tagged IKKεK38A (500 ng). Cells were subjected to ^{32}P metabolic labeling and c-Rel was immunoprecipitated from whole cell extracts using anti-myc antibodies. Immunocomplexes were resolved by SDS-PAGE and radioactivity incorporation was measured by autoradiography. **C)** Flag-IKKε, Flag-IKKβ or myc-IKKα were *in vitro* transcribed/translated in rabbit reticulocyte lysates and immunoprecipitated (IP) with anti-flag or anti-myc antibodies. *In vitro* kinase assays were performed with the immunoprecipitated kinases and purified GST-cRel transactivation domain fusion protein (a.a 422-588), GST-IκBα (a.a. 1-55) or GST alone. Substrates were stained with commassie blue (CB) and ^{32}P incorporation (KA) was measured by autoradiography. *Antibody heavy chain; **Antibody light chain.


A
Myc-cRel - + + + +
Flag-IKKεwt - - - -
Flag-IKKεK38A - - - - +
p-cRel
cRel
ns
Flag
Actin
B
WT
K38A
cRel + + + +
IKKε - + - +
32P cRel
IB: cRel
C
In vitro transcribed and translated kinases:
Empty vector
Flag-IKK ε
Flag-IKK β
Myc-IKK α
Substrate GST-peptide:
IκBα
cRel
GST
cRel
IκBα
GST
KA
CB
IP:
α-Flag
α -Flag
α-Flag
α- myc

treated immunoprecipitated phospho-cRel with phosphatase. However, the phosphatase treatment abolished the recognition of cRel by the antibody in western blotting (data not shown). To further study the relation between IKKε and c-Rel, we tested by immunoprecipitation if these two proteins interact together. Unfortunately, we were unable to see an interaction from these experiments, but this does not exclude that the interaction between c-Rel and IKKε exists.

2. IKKε and TBK-1 directly phosphorylate c-Rel TAD *In vitro*.

To verify whether c-Rel is directly phosphorylated by IKKε or TBK-1, or whether this phosphorylation is the indirect consequence of the activation of another kinase, in vitro kinase assay was performed using recombinant flag-tagged IKKε WT, IKKε K38A, or IKKβ WT or myc-tagged IKKα WT produced in the rabbit reticulocytes lysate system (Promega) as described in the material and method section.

Phosphorylation of the RHD or the TD of p65 and c-Rel was shown to participate in their regulation through increased binding with coactivators, increased DNA binding or transactivation potential (31). IKKβ and TBK-1 was shown to potentiate p65 activity through phosphorylation of serine 536, which is located in the transactivation domain. By homology, our hypothesis was that the transactivation domain of c-Rel might be targeted for phosphorylation by IKKε and/or TBK-1. While preparing this report, Buss et al suggested that multiple kinases including IKKα, IKKβ, IKKε, TBK-1 and another unidentified kinase have the potential to phosphorylate p65 on sernie 536 constitutively and following IL-1 stimulation (22). Thus, in order to answer the question whether c-Rel is a direct substrate for IKKε or TBK-1, *in vitro* kinase assays was performed using as substrate a purified GST-c-Rel-TD protein, which consist of glutathione S-transferase (GST) and c-Rel TD (a.a.422-588) fusion protein, or GST alone as negative control (see figure 8 for description of the method).

A purified GST-IκBα (aa 1-55) substrate (96) was used as positive control for all IκB kinases (IKK) activity. Immunoprecipitated kinases were incubated with substrates in a kinase assay reaction mix in the presence of ^{32}P-labeled ATP. Proteins were separated by SDS-PAGE, stained with Coomassie blue and exposed to autoradiography. The positive control GST-IκBα was phosphorylated by all three IKK kinases but, GST-c-

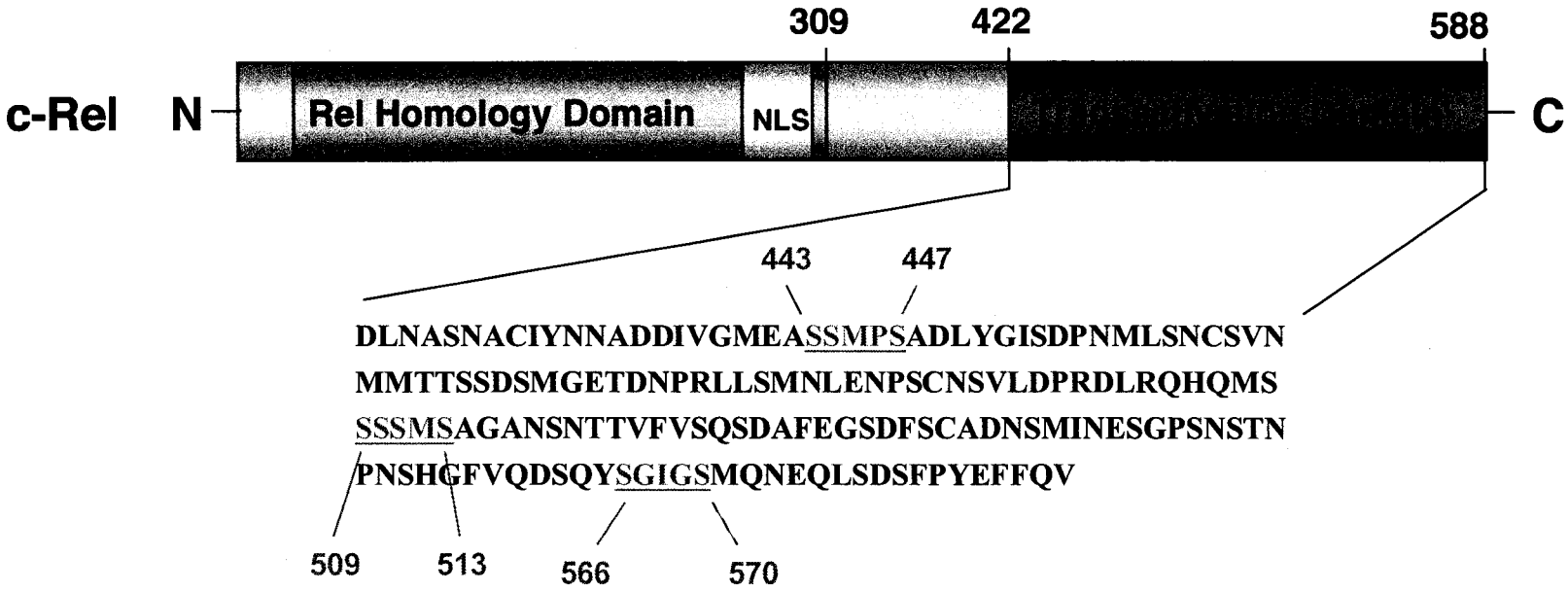
Rel-TD was phosphorylated only by IKKε and IKKβ (figure 9 panel C). As shown in figure 14 IKKεWT, but not IKKε K38A, has the potential to phosphorylate GST-c-Rel TD *in vitro* (lanes 9 and 10). Expression of the *in vitro* transcribed/translated flag-tagged IKKε WT or IKKε K38A was revealed by immunoblotting with anti-flag antibody of the corresponding part of the gel transferred onto nitrocellulose membrane. These results indicate that IKKε and IKKβ, but neither IKKε K38A nor IKKα, directly phosphorylate the transactivation domain of c-Rel *in vitro*.

Expression of TBK-1 was also performed in the reticulocytes lysate system, however no kinase activity was detected. Thus, in order to analyze the activity of TBK-1, His-tagged recombinant TBK-1 kinase was produced in sf9 insect cells using the baculovirus system. After purification of the TBK-1 recombinant kinase produced in insect cells, an *in vitro* kinase assay was performed using GST-c-Rel-TAD and a GST-IRF-3 protein substrates (positive control) (159). The results of the kinase assay (data not shown) indicate that c-Rel TAD is also directly phosphorylated by recombinant TBK-1. Taken together, these results demonstrate that IKKε and TBK-1 as well as IKKβ kinases, have the potential to directly phosphorylate the transactivation domain of c-Rel *in vitro*.

Figure 10. Schematic representation of c-Rel showing the amino acid sequence of its transactivation domain

Schematic representation of c-Rel showing the amino acid sequence of its transactivation domain. The consensus for IKKε is beleived to be a SXXXS motif where the last serine is the target for phosphorylation. In the transactivation domain of c-Rel, there are three potential sites for IKKε phosphorylation. The serine residues that are potentially targeted (447, 513 and 570) are highlighted in red.

SXXXS


309
422
588
c-Rel
N
Rel Homology Domain
NLS
C
443
447
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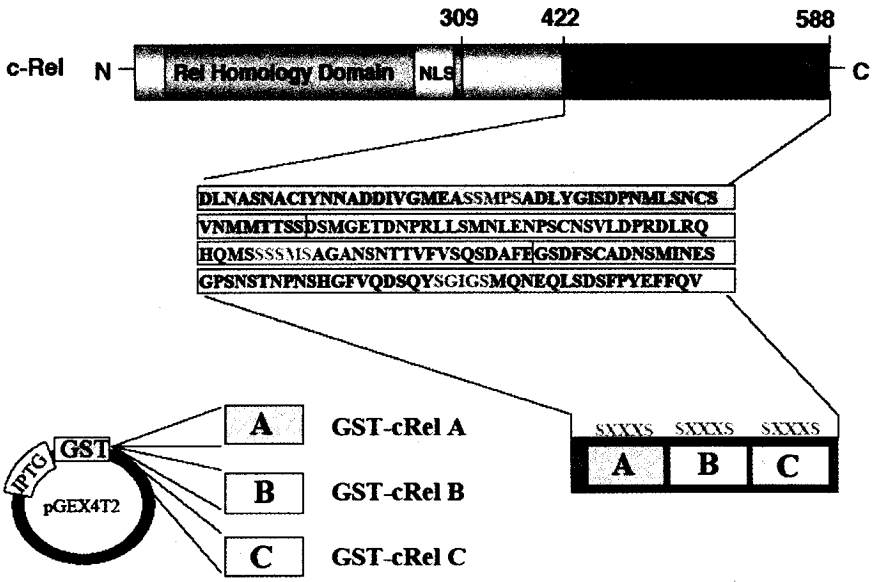
SSSMSAGANSNTTVFVSQSDAFEGSDFSCADNSMINESGPSNSTN
PNSHGFVQDSQYSGIGSMQNEQLSDSFPYEFFQV
509
513
566
570

Figure 11. IKKε phosphorylates cRel in region A and C in the transactivation domain. A) Schematic representation of c-Rel amino acid sequence showing smaller peptides c-RelA (a.a 422- 472), c-RelB (a.a. 473-522) and c-Rel C (a.a. 523-588), each containing one potential consensus site for IKKε/TBK-1 phosphorylation, designed in the transactivation domain. **B)** *In vitro* transcribed/translated Flag-IKKε, Flag-IKKβ or myc-IKKα were immunoprecipitated (IP) with anti-flag or anti-myc antibodies. *In vitro* kinase assays were performed with the immunoprecipitated kinases and purified GST-cRel TD, -c-Rel A, -c-Rel B, –c-Rel C, -IκBα (a.a. 1-55) (positive control) fusion proteins or GST alone (negative control). Substrates were stained with commassie blue (CB) and ^{32}P incorporation (KA) was measured by autoradiography.

A


309
422
588
c-Rel
N
Rel Homology Domain
NLS
C
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GPSNSTNPNSHGFVQDSQYSGIGSMQNEQLSDSFPYEFFQV
SXXXS
SXXXS
SXXXS
A
B
C
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GST
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GST-cRel A
B
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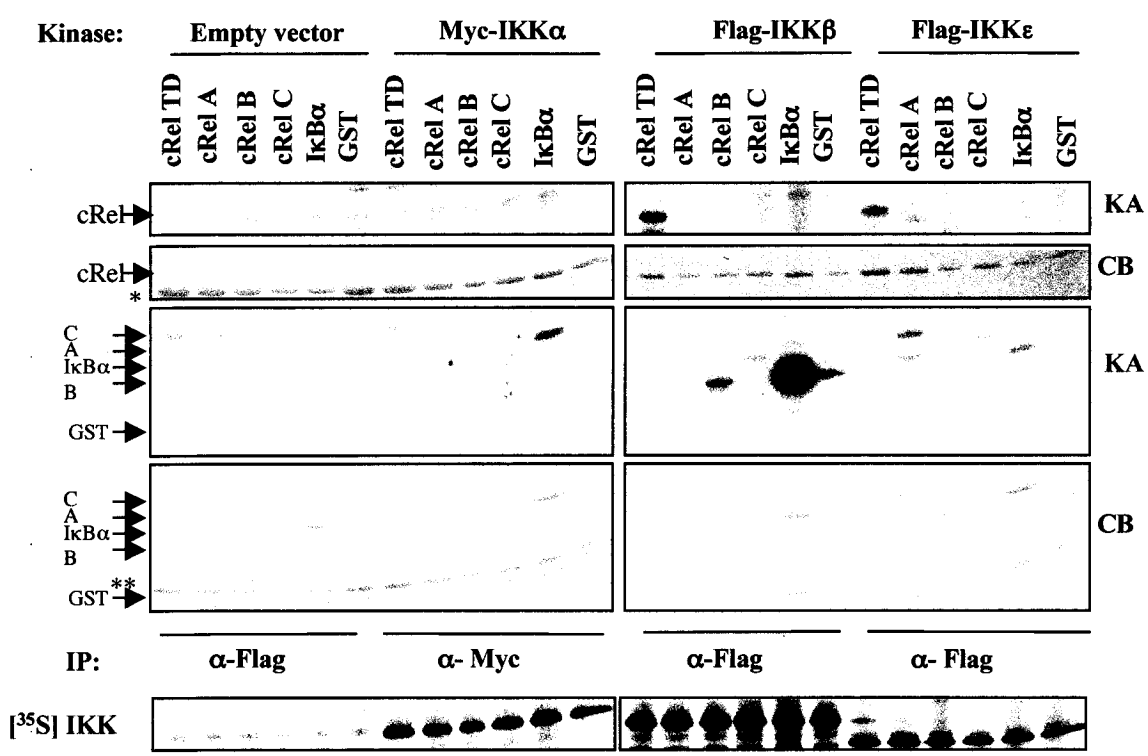
C
GST-cRel C

B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Myc-IKKα
Flag-IKKβ
Flag-IKKε
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cRel A
cRel B
cRel C
IκBα
GST
cRel
cRel
*
KA
CB
C
A
IκBα
B
GST
**
IP:
α-Flag
α- Myc
α-Flag
α- Flag
[35S] IKK

3. IKKε and TBK-1 phosphorylate c-Rel at two sites in the transactivation domain.

Based on the similarity of IKKε and TBK-1 kinase activity with IKKβ (161) as well as on studies of IKKε and TBK-1 kinase activities (78, 91), the consensus for IKKε/TBK-1 phosphorylation is suggested to be an amino acid sequence of Ser-X-X-X-Ser (Ser = serine, X = any amino acid) where the last serine residue would be the phosphoacceptor site. Analysis of the amino acid sequence of the c-Rel transactivation domain revealed the existence of three IKKε/TBK-1 consensus sequences (figure 10). To determine which of these sites are targeted by IKKε and TBK-1, smaller peptides of c-Rel TAD were designed and named A, B and C, each of them containing only one consensus site (figure 11 panel A). GST fusion proteins of each of the smaller peptides were generated and *in vitro* kinase assay with recombinant IKKε WT, IKKβ WT, IKKα WT or TBK-1WT was performed. Interestingly, the results obtained in figure 11 panel B indicate that c-Rel is differentially phosphorylated in its transactivation domain by IKKε or IKKβ. Similar results to IKKε were obtained with recombinant TBK-1 (data not shown). Regions A and C are phosphorylated by IKKε and TBK-1, whereas only region B is phosphorylated by IKKβ.

4. Multiple serine residues in c-Rel transactivation domain are phosphorylated by IKKε *in vitro*.

In order to identify if one specific serine residue is essential for IKKε-mediated phosphorylation of c-Rel transactivation domain, single serine to alanine mutations were created in the GST-c-Rel TD fusion protein. All 6 serine residues found in the three IKKε consensus of c-Rel transactivation domain were mutated (figure 12 panel A). Using an *in vitro* transcribed/translated recombinant flag-tagged IKKε, *in vitro* kinase assay was performed with these GST-c-Rel TD serine to alanine mutants. As we observe in figure 12 panel B, all of these single amino acid mutants are still phosphorylated by IKKε. This is indicating that none of theses serine is essential for the subsequent phosphorylation of other serine residues and that a single amino acid mutation is not sufficient to abolish c-

Rel phosphorylation by IKKε *in vitro*. These results *are* consistent with the previous results (figure 11 panel B indicating that IKKε phosphorylates two parts of c-Rel transactivation domain, regions A and C.

In order to identify which serine residues of the consensus present in regions A and C is the target for IKKε, serine to alanine mutations of both serines in the consensus of the GST-c-Rel peptides A and C were generated (figure 13). Using an *in vitro* transcribed/translated recombinant flag-tagged IKKε WT, kinase assay was performed with GST-c-Rel peptide A WT, or S443A or S447A and GST-c-Rel peptide C WT or S566A or S570A. Although a decrease in the IKKε-induced phosphorylation is observed (figure 14) with the serine to alanine mutant S447A of GST-c-Rel peptide A when compared with the corresponding WT (compare lane 4 with lane 2) as well as with the serine to alanine mutants S566A or S570A of GST-c-Rel peptide C (compare lanes 6 and 7 with lane 5), the phosphorylation signal is not completely abolished. Taken together, these results suggest that serine 447 in region A and serines 566 and 570 in region C are phosphorylated by IKKε *in vivo*. Thus, more than one serine residue is phosphorylated by IKKε *in vivo*. These indications do not exclude the possibility of the existence of other phosphorylation site for IKKε that are not in a SXXXS motif.

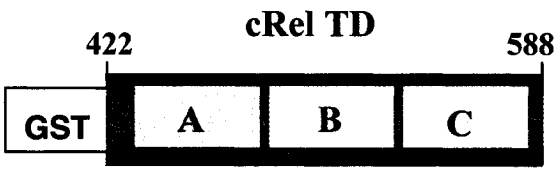
5. IKKε and TBK-1 induces nuclear translocation.

In resting cells, c-Rel is maintained in the cytoplasm in its inactive form, bound to the IκB inhibitory proteins. Once activated, c-Rel translocates to the nucleus where it promotes gene transcription. In order to study the effect of this phosphorylation on c-Rel behavior, we first looked at its subcellular localization in the presence or absence of IKKε or TBK-1. HEK293T cells were cotransfected with a myc-tagged c-Rel encoding construct with a plasmid expressing flag-tagged IKKε WT or flag-tagged IKKε K38A or an empty vector. As endogenous IκBα is not present in sufficient amounts to sequester overexpressed c-Rel into the cytoplasm (data not shown), an IκBα construct was cotransfected in all conditions, in a 1:1 ratio with the c-Rel construct. After 24h of expression, cells were fixed and stained with a mixture of Cy3-labeled anti-myc and FITC-labeled anti-flag antibodies. Nuclei were stained with Hoechst 33342. As shown in

Figure 12. Several serine residues in c-Rel transactivation domain are targeted by IKKε.

A) Schematic representation of GST-c-Rel TD amino acid sequence showing single mutation in c-Rel transactivation domain (aa 422-588). **B)** *In vitro* transcribed/translated Flag-IKKε was immunoprecipitated (IP) with anti-flag antibody. *In vitro* kinase assays were performed with the immunoprecipitated kinases and purified GST fusion proteins GST-cRel TD WT, -c-Rel TD S443A, , -c-Rel TD S447A, -c-Rel TD S566A, -c-Rel TD S570A, -IκBα (a.a. 1-55) (positive control) or GST alone (negative control). Substrates were stained with commassie blue (CB) and ^{32}P incorporation (KA) was measured by autoradiography.

A



GST-cRel 422-588 WT	SXXXS	SXXXS	SXXXS
GST-cRel S443A	**A**XXXS	SXXXS	SXXXS
GST-cRel S447A	SXXX**A**	SXXXS	SXXXS
GST-cRel S509A	SXXXS	**A**XXXS	SXXXS
GST-cRel S513A	SXXXS	SXXX**A**	SXXXS
GST-cRel S566A	SXXXS	SXXXS	**A**XXXS
GST-cRel S570A	SXXXS	SXXXS	SXXX**A**

B

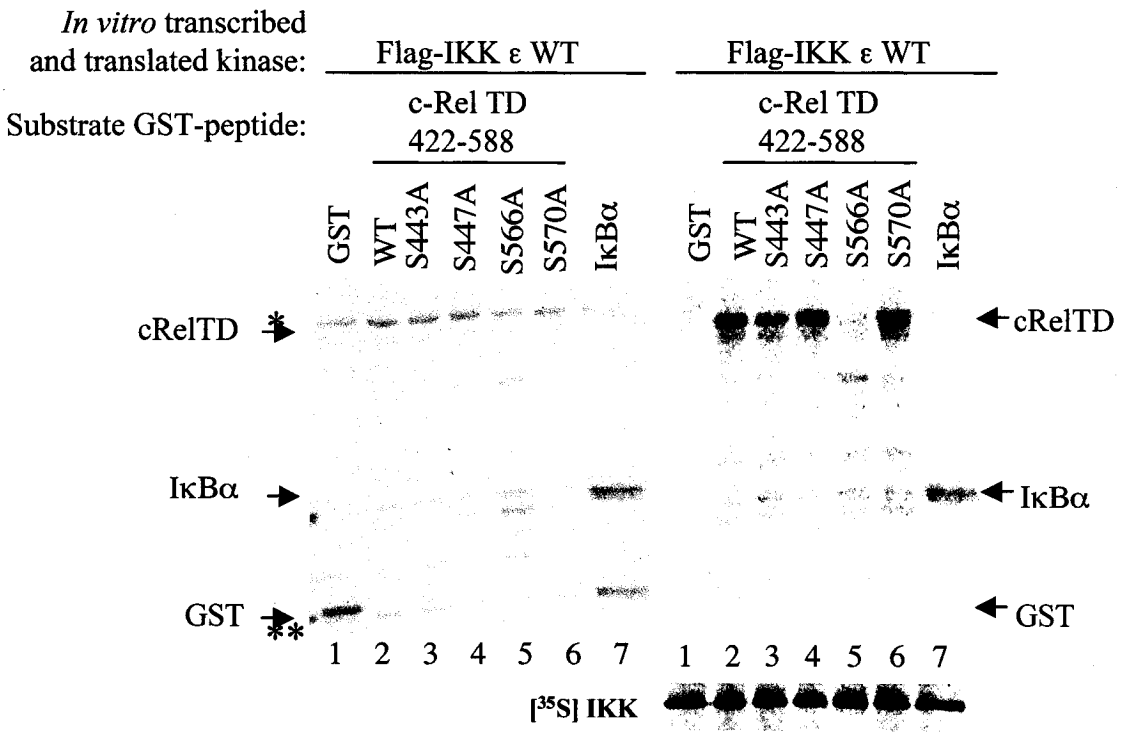
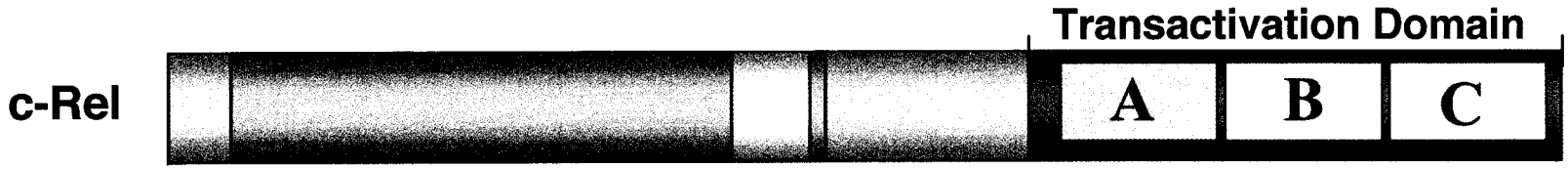


Figure 13. Several serine residues in c-Rel transactivation domain are targeted by IKKε.

Schematic representation of c-Rel amino acid sequence showing smaller peptides c-RelA (a.a 422- 472), c-RelB (a.a. 473-522) and c-Rel C (a.a. 523-588), each containing a single serine to alanine mutation in the SXXXS motif.



GST-cRel peptide A WT	SXXXS
GST-cRel peptide A S443A	AXXXS
GST-cRel peptide A S447A	SXXXA

GST-cRel peptide B WT	SXXXS
GST-cRel peptide B S509A	AXXXS
GST-cRel peptide B S513A	SXXXA

GST-cRel peptide C WT	SXXXS
GST-cRel peptide C S566A	AXXXS
GST-cRel peptide C S570A	SXXXA

Figure 14. Single mutation of the serine residue into alanine in the SXXXS motif of the smaller peptides is not sufficient to inhibit IKKε-mediated posphorylation of c-Rel

In vitro transcribed/translated flag-IKKε WT or K38A was immunoprecipitated (IP) with anti-flag antibody. *In vitro* kinase assays were performed with the immunoprecipitated kinases and purified GST fusion proteins GST-cRel TD WT, -c-RelA S443A, , -c-RelA S447A, -c-RelB S509A, -c-RelB S513A, -c-RelC S566A, -c-RelC S570A, -IκBα (a.a. 1-55) (positive control) or GST alone (negative control). Substrates were stained with commassie blue (CB) and ^{32}P incorporation (KA) was measured by autoradiography.

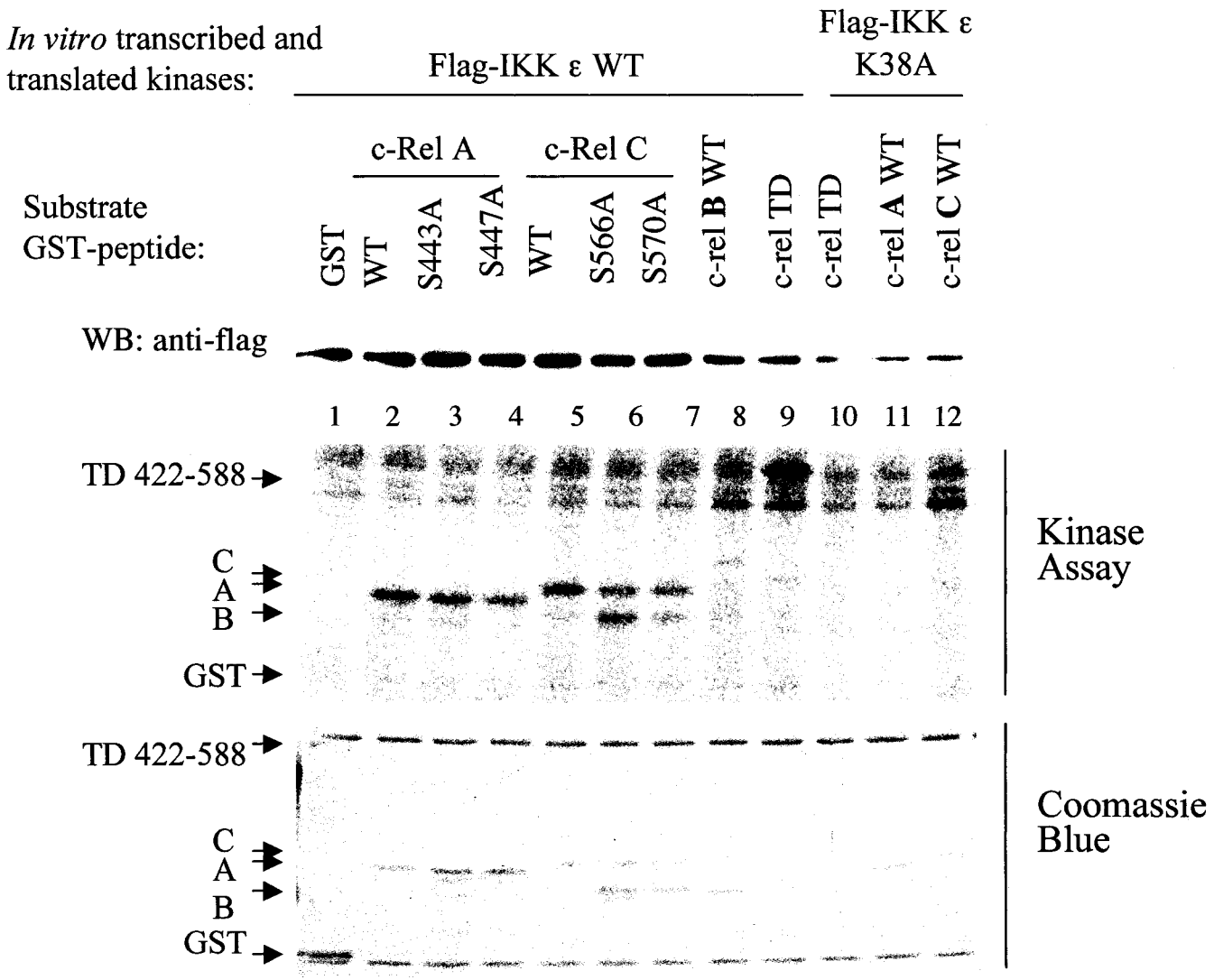

In vitro transcribed and translated kinases:
Flag-IKK ε WT
Flag-IKK ε K38A
c-Rel A
c-Rel C
Substrate GST-peptide:
GST
WT
S443A
S447A
WT
S566A
S570A
c-rel B WT
c-rel TD
c-rel TD
c-rel A WT
c-rel C WT
WB: anti-flag
1 2 3 4 5 6 7 8 9 10 11 12
TD 422-588
C
A
B
GST
Kinase Assay
TD 422-588
C
A
B
GST
Coomassie Blue

Figure 15. IKKε activity induces c-Rel nuclear translocation.

293T cells were co-transfected with plasmids encoding myc-tagged c-Rel and either an empty vector or flag-IKKεWT or flag-IKKεK38A expressing plasmids. Cells were fixed and stained with Cy3-labelled anti-myc (red), FITC-labelled anti-flag (green) and nuclei were revealed with Hoechst 33342 (blue). Percentage of cells expressing c-Rel in the nucleus was determined by counting cells expressing myc-cRel and Flag-IKKε(when transfected). Three 40X fields were counted for each condition.

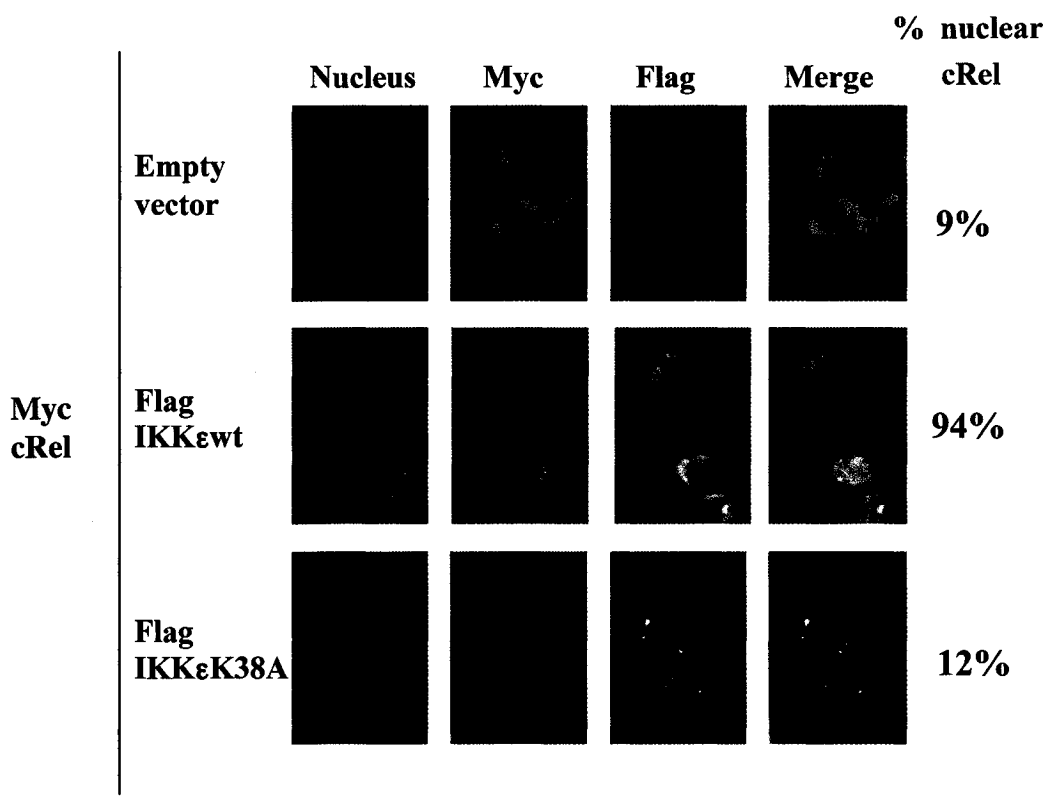

% nuclear cRel
Nucleus
Myc
Flag
Merge
Empty vector
9%
Myc cRel
Flag IKKεwt
94%
Flag IKKεK38A
12%

figure 15, when c-Rel is coexpressed with the empty vector, it remains mostly cytoplasmic with only 9% of the transfected cells showing exclusively nuclear c-Rel. Interestingly, when IKKε WT flag is coexpressed, c-Rel translocates to the nucleus in 94% of the cells coexpressing c-Rel and IKKε. When the K38A kinase dead variant of IKKε is expressed with c-Rel, the later remains mostly cytoplasmic as when it is expressed with the empty vector, with only 12% of the transfected cells showing exclusively nuclear c-Rel. These results clearly indicate that the kinase activity of IKKε is sufficient to induce c-Rel nuclear translocation. Similar results have been observed with the TBK-1 kinase (data not shown).

6. IKKε induced c-Rel nuclear translocation is independent of IκBα phosphorylation and IKKβ activity.

IKKε and TBK-1 are known to phosphorylate IκBα *in vitro* and *in vivo*, but only on serine 36 and this phosphorylation does not lead to IκBα degradation (18, 134, 135, 161, 174), as the IKKβ-mediated phosphorylation of both serines 32/36 does. In order to verify whether this nuclear translocation event was dependent on either direct or indirect IκBα phosphorylation, HEK293T cells were cotransfected with a flag-tagged IKKε WT encoding construct and a 1:1 ratio of myc-tagged c-Rel and wild type IκBα or IκBα 2N variant encoding plasmids. The IκBα 2N mutant harbor mutation of serines 32 and 36 into alanines that cannot be phosphorylated and this construct acts as a dominant negative (10). After 24h of expression, cells were fixed and stained with a mixture of Cy3-labeled anti-myc and FITC-labeled anti-flag antibodies. Nuclei were stained with Hoechst 33342. As shown in panel A of figure 16, IκBα 2N does not impede IKKε-mediated c-Rel nuclear translocation. In the presence of IκBα or IκBα 2N, c-Rel is mostly nuclear when IKKε WT is expressed. In the presence of IκBα WT, 94% of the IKKε-expressing cells showed exclusively nuclear c-Rel compared to 91% in the cells transfected with IκBα 2N. These results indicate that IKKε mediates c-Rel nuclear translocation independently of IκBα phosphorylation.

To further analyze the phosphorylation status of IκBα and IκBα 2N in the presence of IKKε, HEK293T cells were transfected with a 1:1 ratio of myc-tagged c-Rel

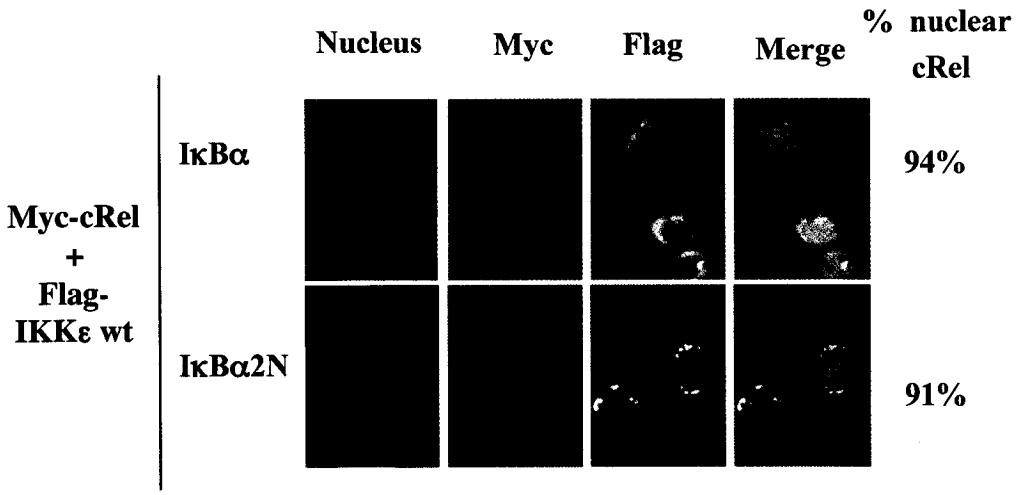
and wild type IκBα or IκBα 2N variant encoding plasmids and a flag-tagged IKKε WT encoding construct or empty vector. Whole cell lysates were separated on SDS-PAGE and immunoblotted using anti-c-Rel, anti-serine 32 phosphorylated IκBα, anti-IκBα, anti-flag and anti-actin antibodies. Interestingly, the results shown in panel B of figure 16, reveal that IκBα is phosphorylated on serine 32 and degraded in the presence of IKKε (lane 3) whereas IκBα 2N is not phosphorylated neither degraded when coexpressed with IKKε (lane 4). Taken together, these results indicate that IKKε expression induce phosphorylation on serine 32 and degradation of IκBα, but not of IκBα 2N and that c-Rel is phosphorylated and translocate to the nucleus independently of IκBα phosphorylation and degradation.

TBK-1 was previously suggested to be upstream of IKKβ in the NF-κB activation pathway (174). On the other hand, both TBK-1 and IKKβ phosphorylate p65 directly on serine residue 536 (17, 18, 22, 52, 130, 148, 149, 193). IKKε has also been suspected to phosphorylate IKKβ, however, the physiological relevance of this event has never been demonstrated (135). Thus, the possible involvement of IKKβ in the IKKε-mediated c-Rel nuclear translocation was further analyzed. An immunofluorescence assay was performed in HEK293T cells transfected with a 1:1 ratio of myc-tagged c-Rel and wild type IκBα encoding plasmids along with a flag-tagged IKKε WT encoding construct or empty vector and an increasing amount of IKKβDN expressing construct. As observed in panel A of figure 17, myc-tagged c-Rel translocates to the nucleus in the presence of IKKε (compare row 1 and 2) and this nuclear translocation is not inhibited by the expression of increasing amounts of IKKβDN (row 3 and 4). However, IKKβDN expression inhibit IKKβ-induced cRel nuclear translocation (row 7 and 8). Panel B is a graphic showing the percentage of transfected cells showing exclusively nuclear c-Rel. These results indicate that IKKε activity induces c-Rel nuclear translocation independently of IKKβ activity. In panel C of figure 17 are shown immunoblots of whole cell lysates from the remaining cells from the immunofluorescence. We observe that both IKKε and IKKβDN are expressed in conditions 3 and 4, where it is undistinguishable on the immunofluorescence pictures, as they are both flag-tagged. The slower migration of phosphorylated cRel is not affected by the expression of IKKβDN (lane 3 and 4). However, the expression of cRel is

Figure 16. IKKε-mediated c-Rel nuclear translocation is independent of IκBα serine 32 and 36 phosphorylation.

293T cells were transfected with plasmids encoding myc-tagged c-Rel and flag-tagged IKKε WT together with plasmids expressing IκBα or IκBα2N. **A)** Cells were fixed and stained with Cy3-labelled anti-myc, FITC-labelled anti-flag and nuclei were revealed with Hoechst 33342. Percentage of cells expressing c-Rel in the nucleus was determined by counting cells coexpressing myc-c-Rel and Flag-IKKε. Quantification represents the average of three 40X fields for each condition. **B)** Whole cell extracts were revealed by immunoblot analysis using anti-cRel, anti-flag, anti-IκBα-phosphoserine 32, anti-IκBα or anti-actin antibodies.

A


Nucleus
Myc
Flag
Merge
% nuclear cRel
Myc-cRel + Flag-IKKε wt
IκBα
IκBα2N
94%
91%

B

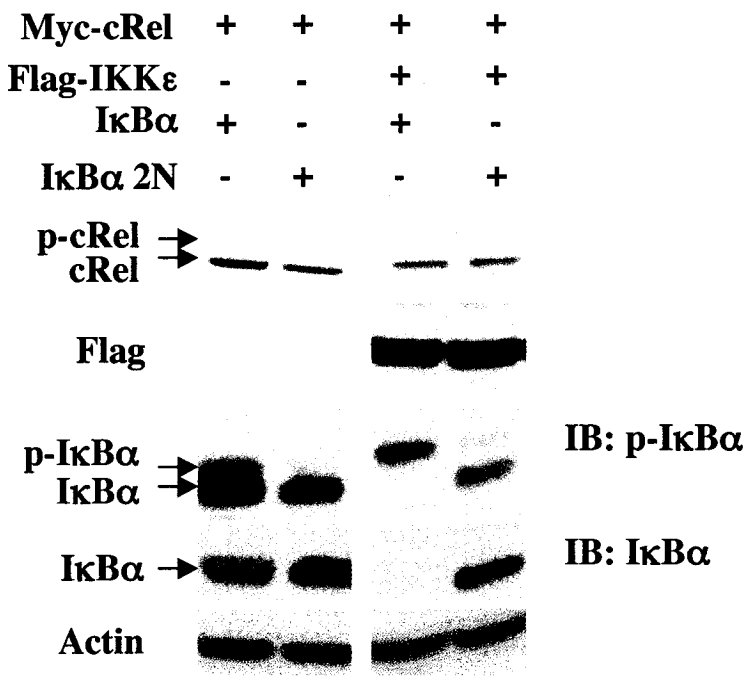
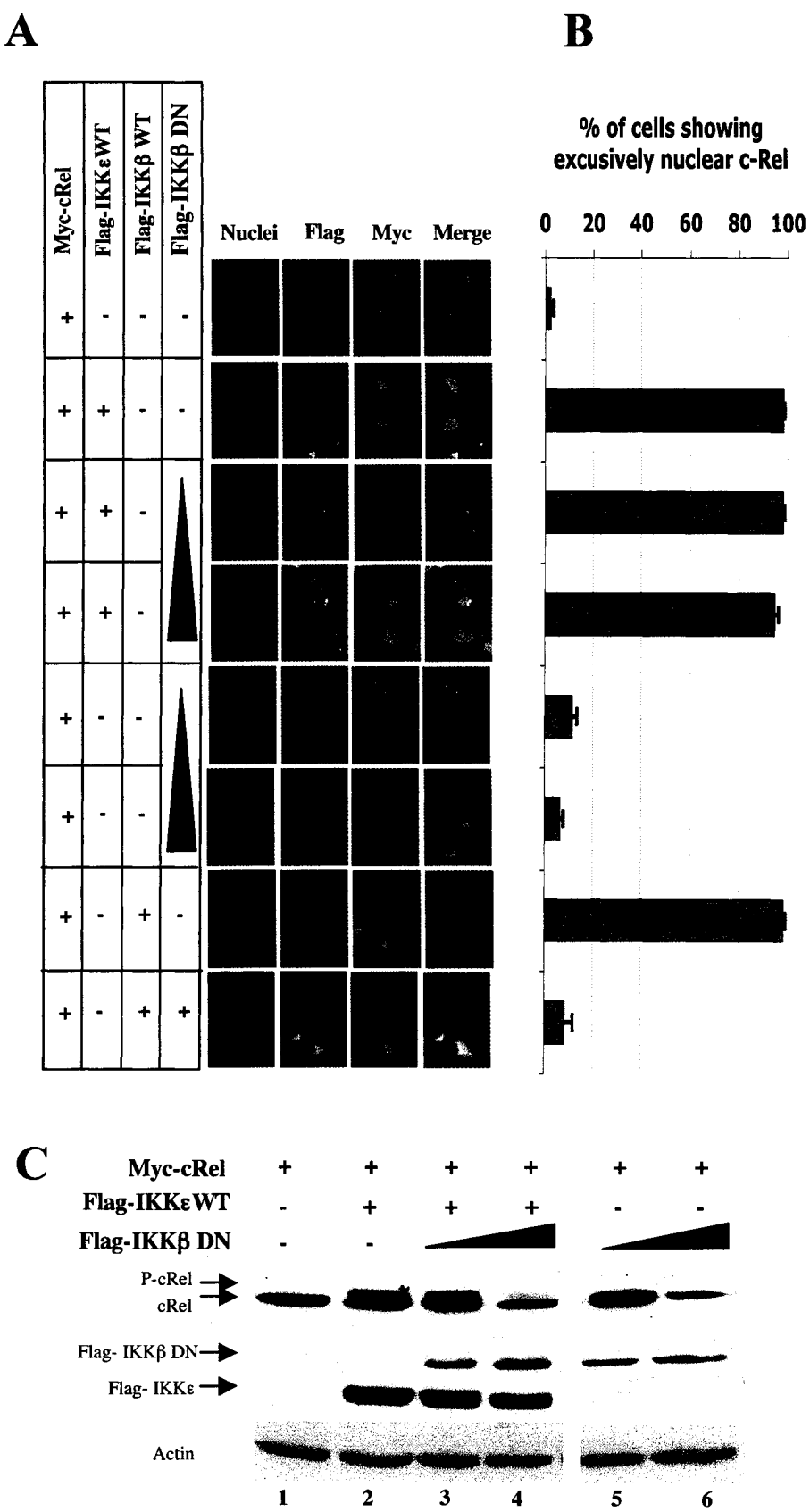

Myc-cRel + + + +
Flag-IKKε - - + +
IκBα + - + -
IκBα 2N - + - +
p-cRel
cRel
Flag
p-IκBα
IκBα
IκBα
Actin
IB: p-IκBα
IB: IκBα

Figure 17. IKKε-mediated c-Rel nuclear translocation is independent of IKKβ activation. 293T cells were cotransfected with plasmids encoding IκBα and myc-tagged c-Rel in a 1:1 ratio with or without flag-tagged-IKKε WT and/or flag-tagged IKKβ WT and/or flag-tagged IKKβDN. **A)** Cells were fixed and stained with Cy3-labelled anti-myc (red), FITC-labelled anti-flag (green) and nuclei were revealed with Hoechst 33342 (blue). **B)** Cells from samples in panel A were counted for c-Rel nuclear localization. Three 40X fields were counted for each condition and only cells expressing Flag-IKKε and IKKβ (when transfected) were counted. Data represented in the graph correspond to the average of the three fields ± SE. **C)** Whole cell extracts were resolved by SDS-PAGE and analysed by immunoblot using anti-c-Rel, anti-Flag or anti-actin antibodies.


A
B
Myc-cRel
Flag-IKKεWT
Flag-IKKβ WT
Flag-IKKβ DN
Nuclei
Flag
Myc
Merge
% of cells showing excusively nuclear c-Rel
0
20
40
60
80
100
C
Myc-cRel
Flag-IKKεWT
Flag-IKKβ DN
P-cRel
cRel
Flag- IKKβ DN
Flag- IKKε
Actin
1
2
3
4
5
6

reduced with the highest concentration of IKKβDN probably due to saturation of the translational machinery and is not specific to phosphorylated cRel as it is also observed in lane 6.

7. S443/447A or S566/570A mutation of region A or C in IKKε consensus of c-Rel TD is not sufficient to abolish IKKε-induced c-Rel nuclear translocation.

In order to identify an essential region in c-Rel transactivation domain for IKKε-induced nuclear translocation, serine to alanine mutations were generated into the sequence of a myc-tagged-c-Rel expression plasmid. According to the previous results suggesting that both serines of the consensus in region A and C are phosphorylated *in vitro* by IKKε (figure 12), those serines in the consensus of regions A (S443/447A) or C (S566/570A) were mutated into alanine in the myc-tagged c-Rel construct (figure 18, panel A). HEK293T cells were cotransfected with a ratio 1:1 of IκBα and myc-tagged c-Rel WT, S443/447A mutant or S566/570A mutant encoding construct with a plasmid expressing flag-tagged IKKε WT or empty vector. After 24h of expression, cells were fixed and stained with a mixture of Cy3-labeled anti-myc and FITC-labeled anti-flag antibodies. Nuclei were stained with Hoechst 33342. As shown in figure 18, panel B, S443/447A mutation of the consensus in region A does not impede IKKε-induced nuclear translocation, neither does S566A/570A mutation of the consensus in region C of c-Rel TD.

8. IKKε increases c-Rel transcriptional activity

In order to assess whether IKKε could have an effect on c-Rel transcriptional activity, reporter gene assays were performed using a promoter specific to c-Rel. The IRF4 promoter was previously characterized and it was shown that the CD28RE of this promoter is specifically responsive to c-Rel/p50 heterodimers and possibly c-Rel homodimers (figure 19 panel A) (158). HEK 293T cells were cotransfected with constant amounts the CD28RE reporter gene, control plasmid p-RLTK for normalization and myc-tagged c-Rel expressing construct along with an increasing amount of a flag-tagged IKKε WT or negative control GFP encoding constructs. As shown in figure 19, panel B, c-Rel transcriptional potential is increased in a dose-dependent manner by IKKε (red bars) as

opposed to the increasing amount of GFP plasmid as negative control (blue bars). This increase in transactivation potential correlates with the phosphorylation pattern of c-Rel observed by immunoblot, when c-Rel is coexpressed with the same increasing amount of flag-tagged IKKε (figure 19, panel C). The results shown in panel C of figure 19 are from an independent experiment where a constant amount of myc-tagged c-Rel construct was cotransfected in HEK 293T cells with an increasing amount of the flag-tagged IKKεWT construct, in the same ratio as for the luciferase assay. After 24h of expression, cells were harvested, lysed and subjected to western blot analysis with anti-c-Rel, anti-flag or anti-actin antibodies. We observe similar results in Jurkat cells transfected with constant amounts of reporter gene pGL3-IRF4 promoter CD28RE, control plasmid p-RLTK for normalization and a 3 fold molar ratio of flag-tagged IKKε WT, flag-tagged IKKε K38A or myc-tagged c-Rel expressing construct. Indeed, in panel D of figure 19 we demonstrate that endogenous c-Rel from Jurkat cells show an increased transaction potential of the CD28RE from the IRF4 promoter in the presence of IKKε WT, but not in the presence of the K38A mutant. These results support the possibility of a more physiologic role for IKKε-mediated c-Rel phosphorylation.

9. IKKε induces proteasome-dependent and -independent c-Rel degradation.

Many transcription factors, such as the oncoprotein c-myc, are regulated through degradation by the proteasome pathway (32, 90, 180). Those transcription factors are activated and degraded to terminate their activity. Very recently, it was shown that p65 is degraded when bound to its promoter and this is an essential event to the termination for NF-κB response (145). Knowing that c-Rel turnover is regulated at the basal level by the ubiquitin-proteasome pathway (29), we asked whether IKKε-induced phosphorylation of c-Rel could be a signal for its degradation by the ubiquitin-proteasome pathway in order to terminate its activity. HEK293T cells were cotransfected with constant amounts of c-Rel and increasing amounts of IKKε WT (figure 20 panel A). We observed that high molecular weight c-Rel proteins are detected on the immunoblot. This observation suggests that c-Rel might be ubiquitinated in the presence of IKKε, thus targeting c-Rel for proteasome-mediated degradation. To verify whether IKKε could induce c-Rel degradation, HEK293T cells were cotransfected with a constant amount of myc-tagged c-

Rel encoding plasmid (2μg) along with an increasing amount of flag-tagged IKKε WT (0.025-2.5 μg) or with IKKε K38A (500μg) encoding constructs. After 24h of expression, cells were harvested and whole cell lysates were analyzed by immunoblotting using anti-c-Rel, anti-flag or anti-actin antibodies. As shown in figure 20 panel B, the signal for c-Rel on the immunoblot is decreased with the increasing amounts of IKKε WT (lanes 1-9) but not with the IKKε K38A (lane 10). In order to determine if the loss of c-Rel signal was due to its degradation by the proteasome, cells were transfected in the same condition as figure 20 panel C, and treated with the MG132 proteasome inhibitor or DMSO as negative control. We observe in figure 20 panel C that with the inhibition of the proteasome, c-Rel is less degraded when coexpressed with lower amounts of IKKε (compare lanes 1-4 with 7-10), but is still degraded with higher amounts of the kinase (compare lanes 5-6 with 11-12). With higher amounts of IKKe (lanes 5, 6 and 11, 12) we observe degradation of c-Rel independently of the MG132 inhibition of the proteasome. By immunofluorescence staining, we observe that with high amounts of transfected IKKε, cells are rounding and the chromatin is condensed (figure 21). These observations suggest that the loss of c-Rel signal on the immunoblot with higher concentrations of IKKε (figure 20, panel C, lanes 5, 6 and 11, 12) is not specific to c-Rel and might be the result of chromatin condensation.

Figure 18. Mutation of the both serine residue into alanine in the SXXXS motif of region A or C in the myc-tagged c-Rel is not sufficient to inhibit IKKε-induced c-Rel nuclear translocation

A) Schematic representation of double mutation of the serine residues into alanine in a single SXXXS motif of region A or C of c-Rel transactivation domain. Mutant A of myc-tagged c-Rel has S443/447A double mutation, mutant B of myc-tagged c-Rel has S509/513A double mutation and mutant C of myc-tagged c-Rel has S566/570A double mutation. **B)** HEK293T cells were transfected with myc-tagged c-Rel constructs: WT, S443/447A or S566/570A along with either empty vector or flag-tagged IKKε WT expressing construct. Cells were fixed and stained with Cy3-labelled anti-myc (red), FITC-labelled anti-flag (green) and nuclei were revealed with Hoechst 33342 (blue).

A

cRel-myc Wild type	SXXXS	SXXXS	SXXXS
cRel-myc mutant A	AXXXA	SXXXS	SXXXS
cRel-myc mutant B	SXXXS	AXXXA	SXXXS
cRel-myc mutant C	SXXXS	SXXXS	AXXXA

B

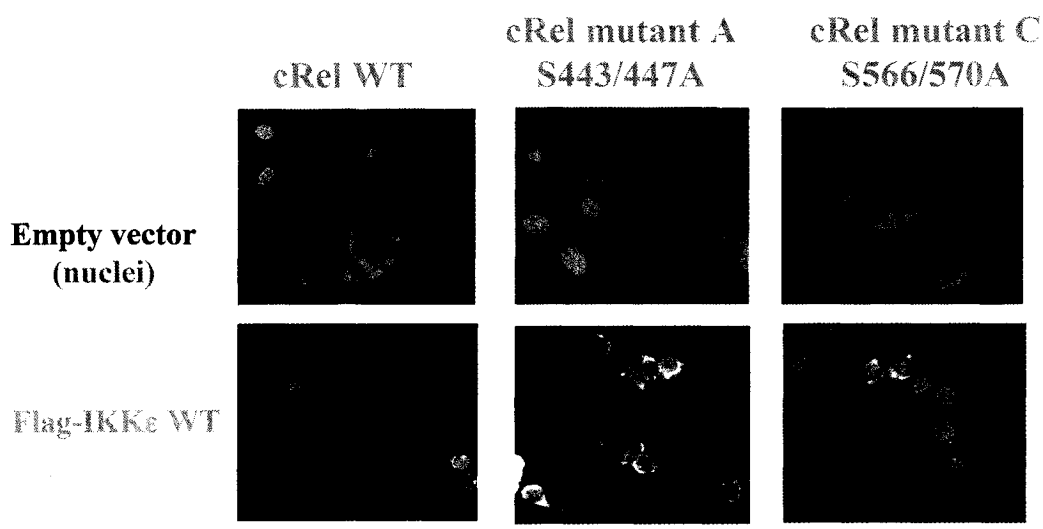
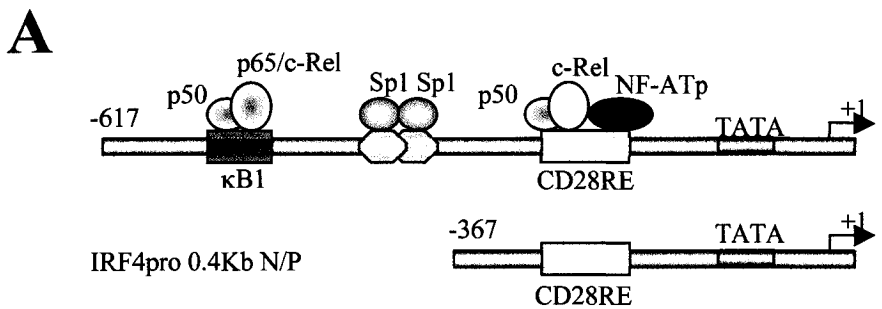
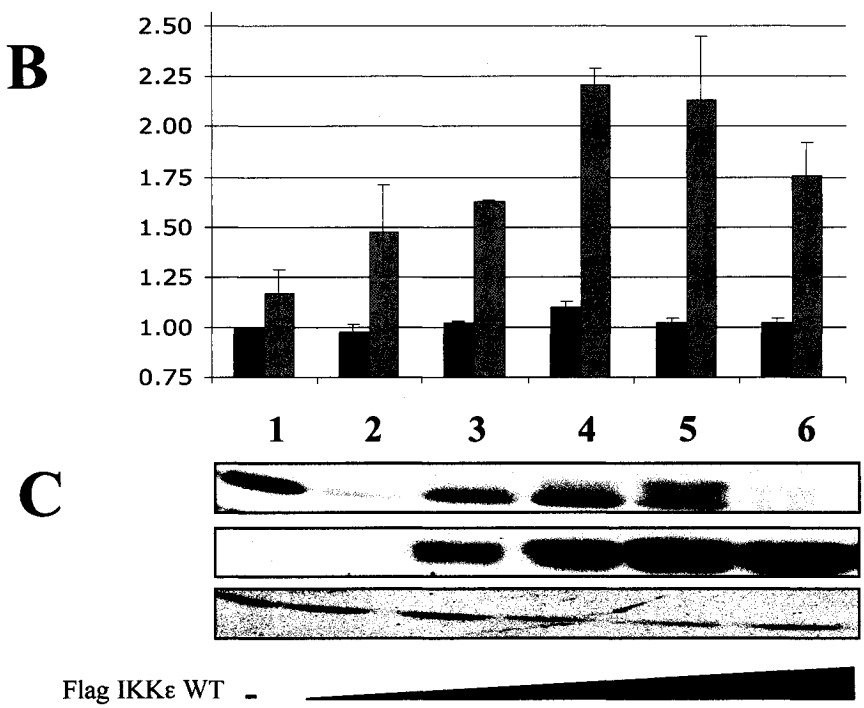


Figure 19. IKKε enhances c-Rel transactivation potential.

A) Schematic representation of the IRF4 promoter. There are two Sp1 sites, a κB binding site (κB1) and a CD28RE that are bound by specific transcription factors. The CD28RE is specific to c-Rel/p50 or c-Rel/c-Rel dimers. Digestions of the IRF4 promoter at –367 generated a portion that comprise only the CD28RE but not the κB1 site and thus is specific to c-Rel and not p65 (158). This –367 to +1 portion of the IRF4 promoter was cloned in the pGL3 reporter plasmid and used for the following luciferase assays. **B)** 293T cells cotransfected with pGL3-IRF4 0.4Kb promoter and plasmids encoding myc-tagged cRel (red bars) or GFP as control (blue bars) with increasing amount of flag-tagged-IKKεwt encoding plasmid. PRL-TK plasmid (renilla luciferase) was cotransfected and used as an internal control. After 24 h transfection, the luciferase activity was measured and expressed as fold activation over the transfection of c-Rel with empty vector after normalization with renilla luciferase activity. **C)** The same ratio of myc-tagged-cRel and flag-tagged-IKKεwt were cotransfected in 293T cells and whole cells extracts were resolved by SDS-PAGE and proteins were revealed by immunoblot using anti-cRel, anti-flag or anti-actin antibodies. **D)** Jurkat T cells were transfected with the IRF4 CD28RE reporter plasmid along with a three fold molar ratio of flag-IKKε WT, flag-IKKε K38A or myc-c-Rel expressing constructs or empty vector as negative control. PRL-TK plasmid (renilla luciferase) was cotransfected and used as an internal control. After 24 h transfection, the luciferase activity was measured and expressed as fold activation over the transfection of c-Rel with empty vector after normalization with renilla luciferase activity.


A
p65/c-Rel
p50
Sp1 Sp1
p50
c-Rel
NF-ATp
-617
TATA
+1
κB1
CD28RE
IRF4pro 0.4Kb N/P
-367
TATA
+1
CD28RE


B
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
1
2
3
4
5
6
C
cRel
IKKε
Actin
Flag IKKε WT -

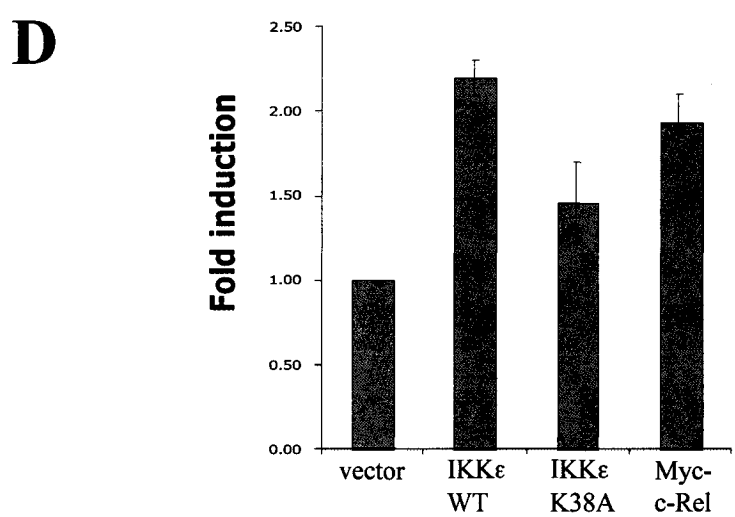
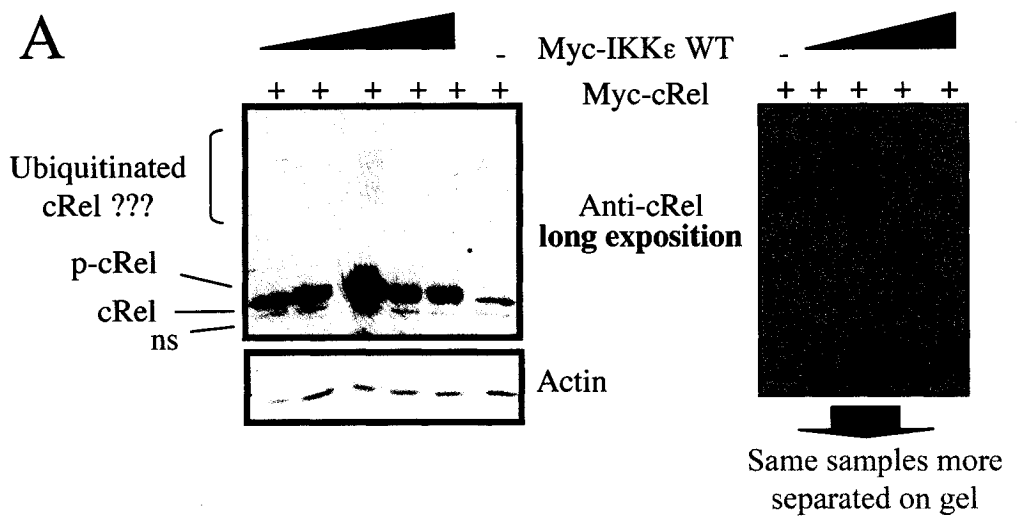
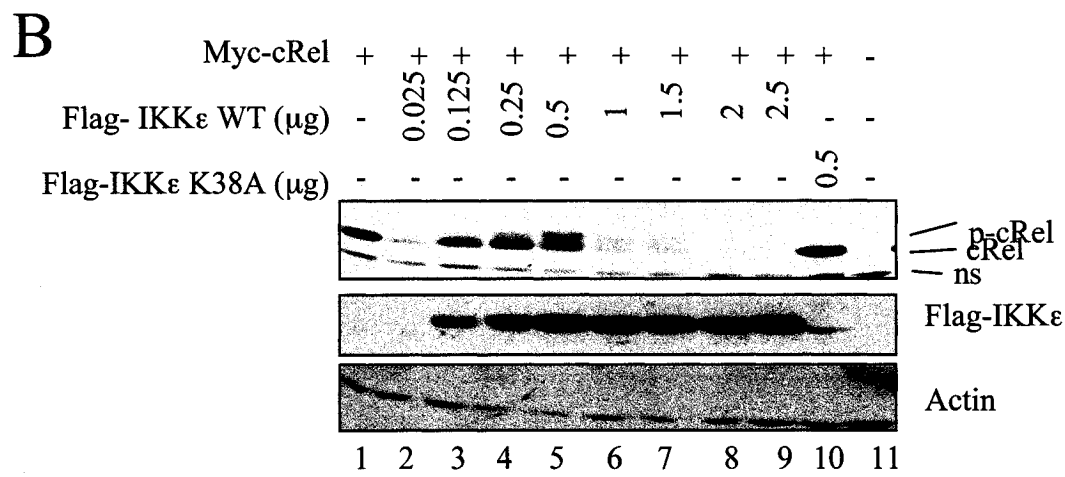

D
Fold induction
2.50
2.00
1.50
1.00
0.50
0.00
vector
IKKε WT
IKKε K38A
Myc-c-Rel

Figure 20. Increasing amount of IKKε expression induces proteasome-dependent and independent cRel degradation.

293T cells were cotransfected with plasmids encoding myc-tagged cRel and an increasing amount of flag-tagged IKKε WT encoding plasmid or empty vector. Cells were either treated with DMSO (control vehicle) or MG132 proteasome inhibitor (5 μM) for 6h. Proteins were resolved by SDS-PAGE and analyzed by immunoblot using anti-cRel or anti-actin antibodies.


A
Myc-IKKε WT
Myc-cRel
Ubiquitinated
cRel ???
p-cRel
cRel
ns
Anti-cRel
long exposition
Actin
Same samples more
separated on gel


B
Myc-cRel
Flag- IKKε WT (μg)
Flag-IKKε K38A (μg)
0.025
0.125
0.25
0.5
1
1.5
2
2.5
0.5
p-cRel
cRel
ns
Flag-IKKε
Actin
1 2 3 4 5 6 7 8 9 10 11

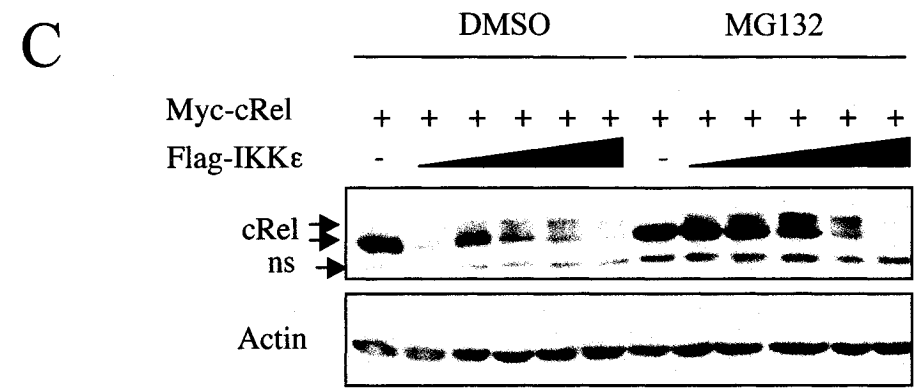
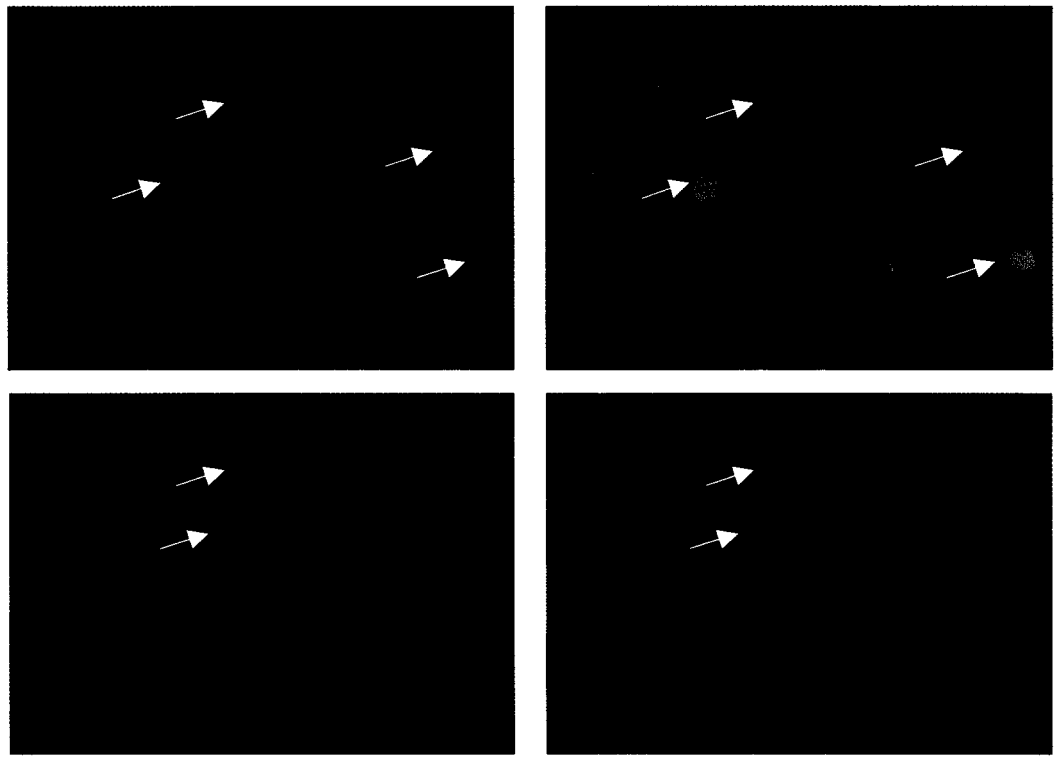

C
DMSO
MG132
Myc-cRel
Flag-IKKε
cRel
ns
Actin

Figure 21. High IKKε expression induces chromatin condensation.

293T cells were cotransfected with plasmids encoding myc-tagged IKKε WT (2μg). After 24h of expression, cells were fixed and stained with Cy3-labelled anti-myc (red) and nuclei were stained with Hoechst 33342 (blue). Cells expressing IKKε WT (right panels, red) are shown with arrows and the corresponding nuclei (blue) are shown in the left panels. The upper panels represent picture taken with a 20X objective and lower panels with 40X objective.



Dapi (nucleus)

Myc-IKKε WT

CHAPTER IV

DISCUSSION

1. Role of IKKε- and TBK-1-induced phosphorylation of c-Rel

It is now well accepted that NF-κB activity is not only regulated at the level of IκB degradation, but that further posttranslational modifications of the NF-κB subunits, such as phosphorylation, are required for full activity (review in (31)). The p65 subunit is phosphorylated by several kinases in response to various stimuli. Among these kinases are the IKKα, IKKβ, IKKε and TBK-1. Although, the physiological effect of the IKKε and TBK-1 mediated phosphorylation of p65 is not fully understood, these two IKK-related kinases were demonstrated to have a role in the activation of other transcription factors, such as IRF3/IRF7, by direct phosphorylation (159). Studies on the role of IKKε in NF-κB activation suggest a special role for IKKε in the immune response (134, 161). Moreover, IKKε is constitutively expressed in lymphoid cells (134, 161). Thus, we hypothesized that IKKε might have a specific role in the NF-κB activation in these cells. Since c-Rel is an NF-κB subunit that is restricted to immune cells, we investigated the effect of IKKε on c-Rel. By coexpression studies, we show that c-Rel migrates more slowly on SDS-PAGE when coexpressed with IKKε and that the migration pattern is unaffected when coexpressed with the kinase dead variant of IKKε. We also observed this slower migration when coexpressed with TBK-1 WT. To validate that this specific pattern of migration is due to the phosphorylation of c-Rel, we performed *in vivo* metabolic labeling with ^{32}P. The results obtained confirmed that c-Rel is more phosphorylated in the presence of IKKε WT but the phosphorylation level is unchanged when coexpressed with the kinase dead variant of IKKε. Other groups have already demonstrated inducible phosphorylation of c-Rel, but only one unknown kinase (TD kinase) was shown to phosphorylate c-Rel in its transactivation domain (46). Martin et al have shown that c-Rel is phosphorylated on serine 471 in response to TNF-α stimulation and this event increases the transcriptional activity of c-Rel. However, the direct kinase has yet to be identified (116, 117). It is also known that c-Rel is phosphorylated and translocates to the nucleus following TCR stimulation by PMA/anti-CD28 (20), where nuclear c-Rel remains detectable until 8 days post TCR stimulation with a peak at 6 days (85). However, the identity of the specific(s) kinase(s) implicated in TCR stimulation-induced c-Rel phosphorylation is currently unknown.

Both IKKε and c-Rel are up-regulated at the expression level following PMA treatment which mimics TCR stimulation (95, 134). This suggests that IKKε-mediated phosphorylation of c-Rel might have a specific role in the sustained NF-κB activation following T cell stimulation.

We have demonstrated that IKKε and TBK-1 phosphorylate c-Rel *in vitro* and *in vivo*. However, we were unable to co-immunoprecipitate c-Rel and IKKε or TBK-1. Knowing that interaction between a kinase and its substrate is transient and might be week, we cannot exclude that IKKε and c-Rel do not interact with one another. To answer directly the question of interaction, BRET (Bioluminescence Resonance Energy Transfer) or yeast two-hybrid analysis should be performed. Although the interaction between c-Rel and IKKε or TBK-1 was not shown, we performed *in vitro* kinase assay with IKKε or TBK-1 to verify if the phosphorylation is a direct event.

As for p65, c-Rel harbors a transactivation domain that is targeted for regulatory phosphorylations; albeit no known kinases have been identified (20, 46, 116, 117). The transactivation domain of c-Rel has been mapped between amino acid 422 to 588 in the C-terminal end (116, 117). Here we show that recombinant IKKε is able to phosphorylate a GST-c-Rel TD (aa 422-588) fusion protein *in vitro*. Since TBK-1 and IKKε are undistinguishable in their substrate specificity, we assayed TBK-1 for c-Rel TD phosphorylation and show that *in vitro* TBK-1 also directly phosphorylates the transactivation domain of c-Rel. These results demonstrated that the transactivation domain of c-Rel is directly phosphorylated by these two kinases, IKKε and TBK-1.

2. Multiple site in c-Rel TD for IKKε-mediated phosphorylation

As opposed to p65, which mutation of serine 536 into alanine is sufficient to abrogate IKKα, IKKβ, IKKε and TBK-1-induced phosphorylation (22), c-Rel appears to have multiple sites for IKKε-mediated phosphorylation. By generation of smaller peptides of c-Rel transactivation domain, we have demonstrated that IKKε and TBK-1 phosphorylate c-Rel at least at two sites, which are comprised in regions A and C. Several serine to alanine mutations have been generated in the transactivation domain of c-Rel in order to identify which serine residues are targeted by IKKε. According to our

results, the single mutation of serine 443 or 447 in region A, serine 509 or 513 in region B or serine 566 or 570 in region C in the full length transactivation domain is not sufficient to abrogate IKKε-mediated phosphorylation *in vitro*. These results suggest that none of those serine residues is essential for subsequent phosphorylation.

Moreover, single mutation in the smaller peptides of c-Rel TD region A or C did not identify a unique phosphorylation site. Despite the small reduction in the phosphorylation signal of some single mutants (c-Rel A S447A, c-Rel C S566A and c-Rel C S570A) when compared with respective wild type peptides, none of them completely abrogated IKKε-induced phosphorylation. Taken together, these results suggest that c-Rel might be phosphorylated to by IKKε to multiple sites. It is also possible that other sites are targeted. To answer the question whether only thoses 3 serines 447, 566 and 570 are targeted, one would need to generate an alanine mutant for all 3 serine residues and perform *in vitro* kinase assay to observe if the phosphorylation signal would be abrogated.

Additionally, myc-tagged c-Rel mutants showed that S443/447A in region A or S566/570A in region C is not sufficient to inhibit IKKε-induced nuclear translocation. These results suggest two possibilities: 1) IKKε-induced nuclear translocation of c-Rel requires the phosphorylation of c-Rel to sites other than those 3 serine residues 447, 566 or 570; 2) phosphorylation of c-Rel in either region A or region C is sufficient to induced nuclear translocation of c-Rel. To answer this question, we should generate a mutant with all 3 serine residues mutated into alanine and assess whether this mutant translocates or not to the nucleus in the presence of IKKε.

Moreover, the shift in phosphorylation is not abrogated by these S443/447A or S566/570A mutation in c-Rel TD (data not shown). Taken together, all of these serine to alanine mutation studies suggest that IKKε mediates the phosphorylation of c-Rel at multiple serine residues and that multiple phosphorylations are required for IKKε-mediated nuclear translocation of c-Rel.

3. IKKε and TBK-1 induce c-Rel nuclear translocation

The effect of direct phosphorylation of NF-κB subunits like p65 and c-rel is variable following the stimuli and the implicated kinases (rewied in (31)). The first step in NF-κB activation is the liberation of NF-κB subunits allowing their translocation to the nucleus. We thus studied the effect of IKKε-mediated phosphorylation on the cellular localization of c-Rel. By immunofluorescence assays in cotransfected cells, we showed that the kinase activity of IKKε and TBK-1 induces c-Rel nuclear translocation. According to studies on c-Rel phosphorylation, this is the first time that a kinase is identified to directly phosphorylate c-Rel in the transactivation domain and to affect nuclear translocation. Most of the studies on NF-κB direct phosphorylation of the transactivation domain show an increase in binding to coactivators or increased transcriptional activity (see table 2 for complete list of references).

4. IKKε-induced nuclear translocation of c-Rel independently of IKKβ activity and Ser32/36 IκBα phosphorylation

Over-expression of IKKε induces serine 32 and 36 phosphorylation of IκBα, as demonstrated by Shimada et al (161). However, IKKε phosphorylates serine 36 only *in vitro*. Moreover, IKKε-/- mice show unchanged degradation of IκBα in response to various activators of NF-κB (93). Here, we demonstrate that IKKε over-expression induce IκBα phosphorylation on serine 32 as well as its degradation. The kinase activity of IKKε also induces c-Rel nuclear translocation, either by direct phosphorylation of c-Rel or by direct or indirect modification of IκBα:c-Rel interaction. Interestingly, the coexpression of a mutated form of IκBα with both serines 32/36 mutated into alanines, which are not phosphorylated either degraded by IKKε expression, did not impede c-Rel nuclear translocation. Moreover, it is not likely that IKKε could act as an IKKβ activating kinase, as the coexpression of a dominant negative form of IKKβ did not abrogate IKKε-induced nuclear translocation. Taken together, these results suggest that IKKε expression induce c-Rel nuclear translocation, which is dependent on the kinase activity. This nuclear translocation of c-Rel is independent of IκBα phosphorylation on serine 32 and 36 as well as independent of IKKβ activity. We propose that IKKε-

mediated phosphorylation of c-Rel could induce a change in its conformation and this would unmask the NLS, which allows nuclear translocation independently of IκBα serine32/36 phosphorylation. In the context of physiological activation of NF-κB, the classical IKKα/β would be activated, which would lead to IκBα ser32/36 phosphorylation and degradation. However, the IKKα/β activation, IKKα/β-mediated phosphorylation and degradation of IκBα are transient and newly synthesized IκBα replenish rapidly the cytoplasmic pool. Interestingly, NF-κB activation is also suggested to induce IKKε expression as it is induced following several stimuli that activate NF-κB, such as LPS, PMA, TNF-α, IFNγ or IL-6 stimulation (161), viral infection, TLR3 and TLR4 stimulation (69) as well as PMA or TCR stimulation (134, 135). We propose that IKKε might be important for the sustained activation of NF-κB following up-regulation at the transcriptional level. This regulation would be independent of IκBα as it would already be re-synthesized.

Interestingly, p65 was shown to translocate to the nucleus independently of IKKα or IKKβ activity, thus independently of IκBα ser32/36 phosphorylation. This particular nuclear translocation is induced by coexpression of PAK1 (51). PAK1 was recently shown to be upstream of IKKε in the viral activation of IRF3, which is induced by IKKε activation (45). PAK1 can be activated by a number of stimuli that all lead to NF-κB activation, such as exposure of epithelial cells to IL1β, TCR stimulation in T cells (189, 196), virus infection (45) and LPS (9). Rac1 and PAK1 are suggested to be upstream of IKKε and TBK-1. Rac and Cdc42hs -which activate PAK1- are already known to regulate NF-κB activity and NF-κB activation by active Ras, Raf-1 or Rac1 requires PAK1 activity (51). Based on these observations, one can propose that the activation of NF-κB through PAK1 might be dependent on IKKε and/or TBK-1. This suggest that IKKε and TBK-1-mediated activation of NF-κB might be dependent of PAK1 but independent of the classical IKKα/β, and IκBα phosphorylation on Ser32/36 and subsequent degradation.

IKKβ induces phosphorylation of IκBα upon TNF-α treatment. Interestingly, p65 is phosphorylated at the time of IκBα upon TNF-α, suggesting a dual role for IKKβ in p65 regulation (148). This phenomenon is quite similar to what we observe in the present

study where IκBα and c-Rel are phosphorylated by the overexpression of IKKε, suggesting also a dual role for IKKε in the regulation of NF-κB. However, it is still unclear how IκBα is regulated by IKKε. Conversely, from this study, we clearly observe that the regulation of c-Rel nuclear translocation mediated by IKKε is independent of IκBα phosphorylation on serine 32/36.

5. IKKε increases c-Rel transcriptional activity

In this study, we demonstrated that overexpressed c-Rel as well as endogenous c-Rel from Jurkat T cells show an increase in the transactivation potential of the CD28RE from the IRF4 promoter when IKKε is expressed. However, without the identification of all serine residues that are targeted, it is not possible to efficiently link the direct phosphorylation of c-Rel in the transactivation domain with the increase in the transactivation. The ideal situation would be to identify all serine residues targeted by IKKε for phosphorylation and mutate these serine residues into alanine. This c-Rel mutant of all targeted residues in the TD would answer the question whether the transactivation is regulated by direct phosphorylation of c-Rel TD. However, this is unlikely possible as there are over 30 serine residues in the transactivation domain of c-Rel. Moreover, it is not excluded that as p65, c-Rel could be phosphorylated to other sites besides the transactivation domain, such as in the RHD. Nevertheless, according to the *in vitro* results of the kinases assays, the increase in the transactivation activity is potentially due to phosphorylation in the transactivation domain.

The mechanistic effect of this increase in transactivation activity was not investigated in the present study, but might be due by example to increased interaction with coactivators or histone acetyltransferases (HAT). By analogy to what is known for p65 (31), increased interaction with HAT such as p300/CBP or PCAF may lead to the acetylation of c-Rel, which could regulate its activity. Indeed, acetylation of non-histone proteins such as transcription factors can have an effect on cellular localization, on interaction with coactivators or on the transcriptional potential (30, 166). Acetylation of histones can activate transcription of NF-κB through the induction of chromatin remodeling, thus giving access to the transcription machinery to achieve its task. Maybe phosphorylation is a signal for interaction with HAT, which in turn acetylates c-Rel.

Indeed, this situation has been observed for p53 where the phosphorylated serine 15 allows acetylation of p53, which is probably a consequence of increased binding to CBP and p300 (113, 147). A similar observation was reported for the retinoblastoma tumor suppressor protein (RB) where the acetylation that controls the protein occured after a phosphorylation event (28). For now, it is not known if c-Rel is regulated by acetylation such as for p65. However, most of the lysine residues known to be acetylated in p65 are conserved among the other member of the NF-κB family including Dorsal from Drosophila. These observations suggest that c-Rel is likely to be regulated by acetylation of those lysine residues just as for p65. Moreover, phosphorylation or acetylation of c-Rel can affect the binding affinity to IκBα.

6. IKKε induced proteasome-dependent and -independent c-Rel degradation

Ending transcription through degradation of c-Rel might be highly possible especially if we consider that IKKε and c-Rel have a role in the late activation of T cells. We know that both IKKε and c-Rel are regulated at the transcriptional level and are upregulated by PMA treatment, which mimics TCR engagement (95, 134). Moreover, IκBα acts as negative regulator of NF-κB rapidly after NF-κB activation. End of transcription by proteasomal degradation of p65 was recently observed (145). Thus, it is likely that c-Rel activity termination would be regulated by proteasomal degradation. Moreover, as many other transcription factors, c-Rel turnover is regulated by the ubiquitin-proteasome pathway. A series of deletion mutants missing portions of the C-terminal transactivation domain showed a reduced susceptibility to proteasome degradation, suggesting a role of the transactivation domain for proteasome-mediated degradation (29). Interestingly, the transcription factor c-myc is regulated by ubiquitination and degradation by the proteasome following activation. Indeed, the F-box protein Skp2 participates in c-Myc proteasomal degradation and acts as a cofactor for c-Myc-regulated transcription (90, 180). Moreover, p300, which is a coactivator for NF-κB, act as a coactivator for p53 and mediates its ubiquitination with its ubiquitine ligase activity (64). These findings suggest that following phosphorylation, c-Rel could also be regulated by association with coactivators harboring ubiquitine ligase activities. In the various steps of the ubiquitination process, at least three ubiquitin ligases (E1, E2 and E3)

are involved (figure 22). There are growing numbers of E3 ubiquitin ligases that function as transcriptional coactivators. It is then possible that IKKε-mediated phosphorylation of c-Rel induce an increased interaction with coactivators such as CBP/p300, which harbor an E3-ubiquitin ligase activity. Binding with coactivators may increase c-Rel transactivation followed by ubiquitination of c-Rel by the coactivator, which would lead to proteasome-mediated degradation.

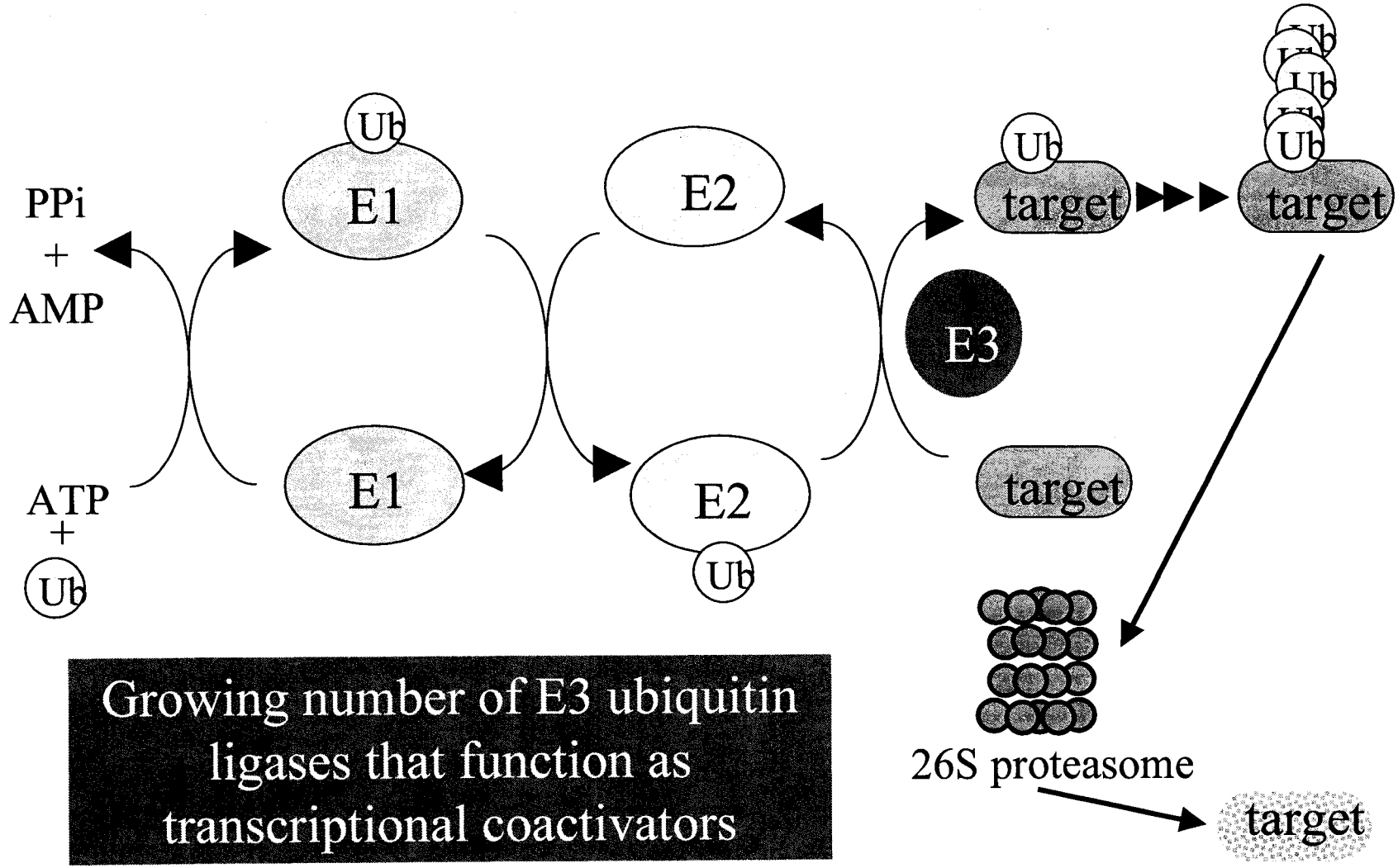
7. Proposed mechanism of action

The proposed model at the molecular level is shown in figure 23. Upon stimulation of the cell by various inducers of NF-κB, the two IKK complexes (IKKα/β/γ and IKKε/TBK-1 containing complex) would be activated. The classical pathway leading to IκBα phosphorylation through the classical IKK would occur, but additional phosphorylation, mediated by IKKε and TBK-1, might be required for full transactivation potential of c-Rel. The fact that IKKε-mediated phosphorylation of c-Rel induced nuclear translocation independently of IKKβ activity or IκBα ser32/36 phosphorylation suggest a role for IKKε in the sustained activation of NF-κB, independently of IκBα. In the early events of NF-κB activation, IκBα degradation would be important to free the NF-κB subunits, but the newly synthesized IκBα would end the early activation of NF-κB rapidly. IKKε-mediated nuclear translocation of c-Rel independently of IκBα would thus be important for the sustained NF-κB activation.

In T cell activation by TCR/CD3 stimulation, the immediate and early phases induce the activation of NF-κB mainly through p65 whereas c-Rel is believed to be a key point for the later phase. The late phase is dependent on TNF-α and it was shown that autocrine and paracrine TNF-α secretion controls the level of c-Rel in T cells (137). From what is known on the regulation of NF-κB, the rapid inducibility as well as the transient nature of NF-κB activation relies on IκBα, which is rapidly degraded and re-synthesized. The sustained activity of NF-κB would therefore be independent of IκBα. As previously mentioned, PAK-1 induces p65 nuclear localization independently of IKKα/β, thus independently of IκBα ser32/36 phosphorylation (51). Moreover, very recently, it was demonstrated that in some cases, PAK1 can associate with ERK2 and this

Figure 22. Schematic representation of the ubiquitination process of proteins targeted to the proteasome.

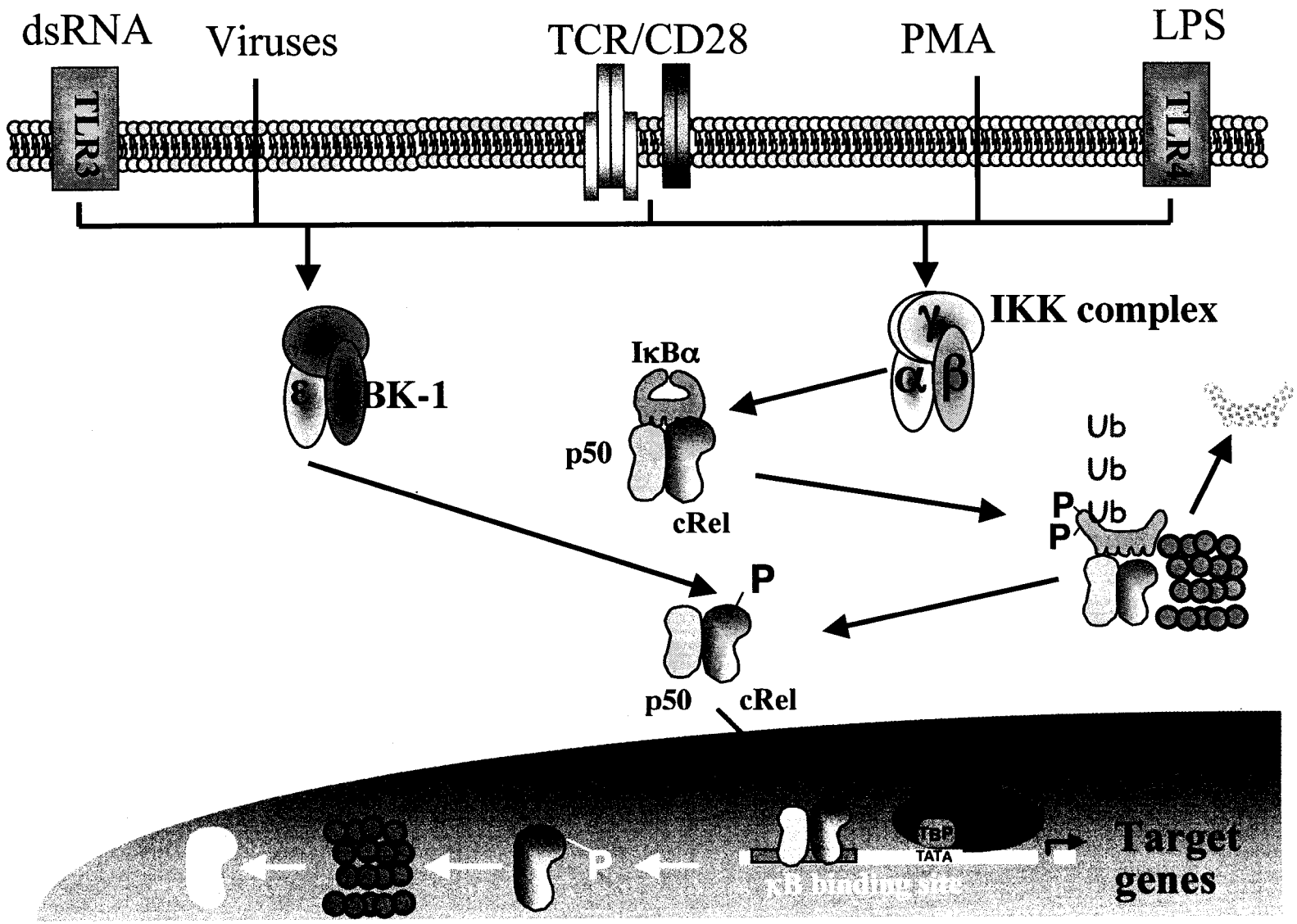
The ubiquitination process involve three or four different enzymes (E1, E2, E3 and sometimes E4). An ubiquitin-activating enzyme (E1) binds ubiquitin and activates it to a thioester, which is then transferred to an ubiquitine-conjugating enzyme (E2). The ubiquitine-protein ligase (E3) helps transfer the ubiquitin to the target substrate to a lysine residue. Multiple ubiquitin molecules are attached to proteins before recognition and degradation by the 26S proteasome. There are increasing number of E3 ubiquitin ligases that function as transcriptional coactivators.



Growing number of E3 ubiquitin ligases that function as transcriptional coactivators

Figure 23. Proposed model of IKKε-mediated c-Rel regulation

Both the classical IKK complex (α/β/γ) and the IKK-related kinases (IKKε and TBK1) are activated upon dsRNA, viruses, LPS, PMA or TCR stimulation. The classical activation of NF-κB through the IKKα/β/γ complex occurs, leading to the early response to NF-κB activation through IκBα Ser32/36 phosphorylation and degradation. The IKK-related kinases might contribute to this early phase but would be more important to the sustained NF-κB activation. In response to these stimulations, IKKε expression increase and would lead to a sustained activation of NF-κB through phosphorylation of c-Rel. The phosphorylation of c-Rel induces its nuclear translocation independently of IκBα Ser32/36 phosphorylation and degradation. The IκBα-independent sustained activation of c-Rel would be terminated through proteasome degradation of nuclear phosphorylated c-Rel.


dsRNA
Viruses
TCR/CD28
PMA
LPS
TLR3
TLR4
IKK complex
γ
α
β
IκBα
p50
cRel
Ub
Ub
Ub
P
P
P
p50
cRel
TBP
TATA
Target genes

facilitates ERK signaling (168). Interestingly, ERK is essential for IL-2 production in the late phase of T cell activation. The inhibition of ERK abrogates IL-2 production in T cells and c-Rel nuclear localization is also inhibited, much more than NF-AT activation (81). According to the literature and based on these findings, we can suggest that in the TCR stimulation, IKKε, PAK1, ERK2 and c-Rel might all be implicated. Thus, we can speculate that TCR stimulation might signal through ERK2 and PAK1 and would activate IKKε. Independently of the classical IKK pathway, IKKε would induce c-Rel phosphorylation and nuclear translocation, which is required for sustained IL-2 production.

However if the sustained NF-κB activity is not terminated by the negative feed back loop of newly synthesized IκBα which bring back the NF-κB subunits to the cytoplasm, how does it ends? As for c-Rel, IKKε is also induced by TNF-α treatment (4). It would therefore make sense to think that IKKε might be important for the sustained activity of NF-κB in the late phase of T cell activation. According to the results obtained from this study regarding the IKKε-induced c-Rel degradation, it would also be reasonable to speculate that this might be a new mechanism for termination of NF-κB activity, independently of IκBα-induced termination.

8. Physiological relevance

Activation of NF-κB has been associated with various types of malignancies. c-Rel is a member of NF-κB that is consistently associated with oncogenic potential. Thus, c-Rel may be an appropriate therapeutic target for certain human lymphoid cancers. Therefore, as c-Rel is overexpressed in a variety of human cancer, and that we know from this study that IKKε can target c-Rel for degradation, it would be reasonable to think that IKKε-mediated phosphorylation of c-Rel might have a role in oncogenesis. IKKε may be a good target to induce by therapeutic drugs to promote the degradation of oncogenic c-Rel. Moreover, if IKKε could be activated by drug treatments, it would also possibly have beneficial effects to treat some virus infection by helping the organism to

produce an effective innate immune response. Also, one could verify the expression and the activity of IKKε in various cancer cells to see if it is down-regulated or inactive.

Several oncoproteins are regulated by proteasome-mediated degradation. For example, the tumor suppressor β-catenin is a coactivator of TCF transcription factors and has the potential to induce TCF activation, which stimulates cell proliferation. All the known oncogenic β-catenin mutants cannot be phosphorylated by the glycogen synthase kinase 3β (GSK3β), which normally phosphorylates β-catenin and targets it for degradation by the proteasome (165). If this is true for coactivators, this could also be the same for transcription factors. It is then possible that mutations in c-Rel that disable it to be phosphorylated by IKKε and consequently degraded by the proteasome, could give an oncogenic potential to those variants, as for the β-catenin mutants. It is also important to mention that v-Rel and c-Rel C-terminal portion are considerably different in their serine residues. Indicating that the target residue for IKKε phosphorylation could be absent in v-Rel, thus possibly impeding its regulation by degradation.

As mentioned previously, c-Rel mediates allergic pulmonary inflammation. Thus, asthmatic reaction could be controlled through the inactivation of c-Rel. This implicates again that the IKKε-mediated degradation of c-Rel becomes an attractive event to stimulate by therapeutic intervention.

9. Contribution to original knowledge

The study presented in this thesis shows for the first time the direct phosphorylation of c-Rel in its transactivation domain by a known kinase. Moreover, IKKε and TBK-1 are newly identified kinases that play an unclear role in NF-κB activation. Here we propose an important role for these kinases in the regulation of NF-κB at the level of direct phosphorylation of c-Rel. Furthermore, we demonstrated that the c-Rel nuclear translocation induced by IKKε is independent of serine 32 and 36 phosphorylation of IκBα as well as from IKKβ activity. This particularity is of interest since it is the first time that nuclear translocation of NF-κB is mediated by an IKK, independently of ser32/36 phosphorylation of IκBα and IKKβ activity. Thus, these

results suggest a completely new pathway for NF-κB activation. Finally, another interesting part of this study is the identification of IKKε-mediated phosphorylation that mediates c-Rel degradation. It is the first time that a study demonstrates the proteasome-mediated degradation of NF-κB following a phosphorylation event, which is mediated by IKKε.

CHAPTER V

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