

REPORT

Nuclear factor (NF)- κ B proteins: therapeutic targets*

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Ann Rheum Dis 2004;63(Suppl II):ii57-ii61. doi: 10.1136/ard.2004.028266

Nuclear factor (NF)- κ B is a key player in the control of both innate and adaptive immunity. NF- κ B proteins are present in the cytoplasm in association with inhibitory proteins called inhibitors of κ B (I κ Bs). On activation by a large plethora of inducers, the I κ B proteins are phosphorylated, ubiquitinated, and subsequently degraded in the proteasomes. Degradation of I κ Bs allows translocation of NF- κ B into the nucleus and bind to their cognate DNA binding sites to regulate the transcription of large numbers of genes including antimicrobial peptides, cytokines, chemokines, stress response proteins, and anti-apoptotic proteins. NF- κ B activity is essential for lymphocyte survival, activation, and mounting normal immune responses. Constitutive activation of NF- κ B pathways is often associated with inflammatory diseases like rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, and asthma. Better understanding of the regulation of NF- κ B will provide a platform for development of specific therapeutic agents targeted towards the inflammatory diseases.

NF- κ B PROTEINS

As most transcription factors, the mammalian NF- κ B family has multiple members including RelA (p65), NF- κ B1 (p50; p105), NF- κ B2 (p52; p100), c-Rel, and RelB^{1,2} (fig 1). These proteins have a structurally conserved N-terminal 300-amino acid region, which contains the dimerisation, nuclear localisation, and DNA binding domains (see fig 1). The c-Rel, RelB, and RelA proteins also have a C-terminal non-homologous transactivation domain that strongly activates transcription from NF- κ B binding sites. On the other hand, the other Rel proteins, such as p50 homodimers, lack the transcription activation domain but still bind to κ B-consensus sites and therefore function as transcription repressors.³ The p50 and p52 proteins are generated by proteolytic processing of their precursors, p105 and p100, respectively.⁴ All members of the NF- κ B family except RelB can form homodimers, as well as heterodimers with one another. The most prevalent activated form of NF- κ B is the heterodimer of subunit p65 associated with either subunit p50 or p52. In contrast, expression of RelB is restricted to specific regions of the thymus, lymph nodes, and Peyer's patches. c-Rel is also confined to the haemopoietic cells and lymphocytes. The transcription of RelB, c-Rel, and p105 themselves is regulated by NF- κ B.

Genes for all five members of the NF- κ B family have been deleted by homologous recombination in mice.⁵ These gene knockout animal models reveal the distinct roles of the NF- κ B proteins in regulation of innate and adaptive immune responses, lymphocyte functions, and cell survival. Mice lacking the p65 subunit exhibit embryonic lethality due to liver degeneration, whereas mice lacking any one of the other four members are immunodeficient without developmental defects. Mice lacking more than one member of NF- κ B family, such as p50^{-/-}/p65^{-/-}, p50^{-/-}/relB^{-/-}, p50^{-/-}/p52^{-/-} show

more severe phenotypes, suggesting a functional redundancy among the NF- κ B family members.

I κ B PROTEINS

NF- κ B exists in the cytoplasm in an inactive form through association with the I κ B proteins, among which the most prominent ones are I κ B α , I κ B β , and I κ B ϵ .³ These regulatory proteins are identified by the presence of multiple ankyrin repeats, a 33-aa motif that mediates protein-protein interactions¹ (see fig 1). Importantly, p100 and p105 also contain similar ankyrin repeats and can function as I κ B-like proteins. In these precursor proteins, the domain containing ankyrin repeats can be proteolytically cleaved and degraded. Another unusual member of the I κ B proteins is Bcl-3 which interacts specifically with p50 and p52 homodimers and can induce expression of κ B regulated genes in contrast with inhibitory functions of other I κ B proteins.^{3,6}

The prevailing view is that I κ Bs retain NF- κ B in the cytoplasm by masking nuclear localisation sequences (NLSs) on NF- κ B subunits. However, recent studies have indicated that the cytoplasmic localisation of the inactive NF- κ B complexes is actually achieved by balancing a continuous shuttle between the nuclear and cytoplasmic compartments.⁷⁻¹² Structural and biochemical experiments have revealed that only one of the two NLSs in the NF- κ B dimer is masked by I κ B α in the NF- κ B·I κ B α complexes, which allows the complexes to shuttle to the nucleus. At the same time, the nuclear export signal (NES) located on the N-terminus of the I κ B α protein functions to constantly expel the NF- κ B·I κ B α complex out of the nucleus. As the export process is more potent than the import process, the nuclear localisation of the inactive NF- κ B·I κ B α complex could only be detected when the nuclear export is blocked by the inhibitor leptomycin B. Like I κ B α , I κ B ϵ is also actively shuttling between the nucleus and cytoplasm.¹³ The advantages of maintaining the inactive NF- κ B complexes in the cytoplasm by the energy consuming shuttling process are currently unclear and need further exploration. In contrast, the NF- κ B·I κ B β complexes are retained in the cytoplasm due to the masking of both NLSs on the NF- κ B dimers by I κ B β .¹⁴ Furthermore, I κ B α but not I κ B β contains a functional NES at its N-terminus that is essential for shuttling the NF- κ B·I κ B α complex out of the nucleus. Biological implications of the constant shuttling of NF- κ B·I κ B complexes between the cytoplasm and nucleus remain elusive.

REGULATION OF NF- κ B ACTIVATION BY I κ Bs AND IKKs

For most known stimuli except ultraviolet irradiation and H₂O₂, degradation of I κ B is an essential step for release of NF- κ B and its subsequent activation.¹⁵ A critical regulatory step in this process is signal induced phosphorylation of I κ B

* This manuscript is an extensive modification of "NF- κ B regulation in the immune system", *Nat Rev Immunol* 2002;2:725-34.

Abbreviations: I κ B, inhibitor of κ B; IKK, I κ B kinase; MEF, murine embryonic fibroblast; NF, nuclear factor; NLS, nuclear localisation sequence; NEMO, NF- κ B essential modulator

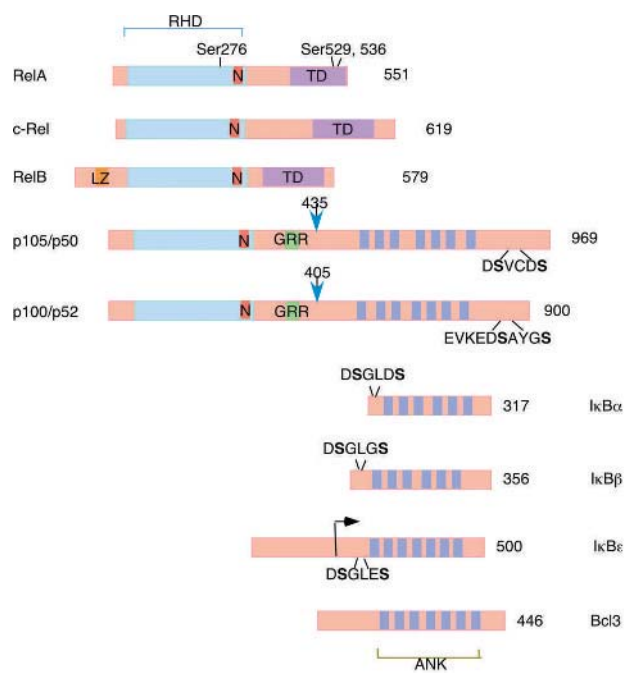


Figure 1 Mammalian nuclear factor (NF)- κ B and inhibitor of κ B (I κ B) protein family members. The number of amino acids in each human protein is shown on right. The NF- κ B family includes five members: RelA (p65), c-Rel, RelB, p105/p50, and p100/p52. They have a structurally conserved N-terminal rel homologous domain (RHD), which contains the dimerisation, nuclear localisation (N), and DNA binding domains. c-Rel, RelB, and RelA proteins also have a C-terminal non-homologous transactivation domain (TD). RelB has an additional leucine zipper motif (LZ). The I κ B family includes I κ B α , I κ B β , I κ B ϵ (two transcripts), and Bcl-3 and is identified by the presence of multiple ankyrin repeats (ANK). The sites of induced phosphorylation of I κ B α , I κ B β , and I κ B ϵ for their degradation are shown. p100 and p105 contain RHD at the N-terminus and ankyrin repeats at the C-terminus. Proteolytic processing of p105 and p100 at 435 and 405 (blue arrowheads), respectively, generates the p50 and p52 proteins. The glycine-rich region (GRR) and the inducible C-terminal phosphorylations are required for processing. Phosphorylation of p65 at Ser276, 529, 536 is important for its transactivation activity.

at specific N-terminal serine residues mediated by I κ B kinases (IKKs). Ser32 and Ser36 phosphorylated I κ B α is then ubiquitinated at lysine 21 and 22 by β -TrCP, which targets it for degradation by the 26S proteasome, thereby releasing NF- κ B dimers from the cytoplasmic NF- κ B·I κ B complex and allowing them to translocate to the nucleus.¹⁵ In the past few years, ubiquitination has attracted increasing attention in the field of NF- κ B as a result of its common involvement in the regulation of signalling processes. Proteolysis associated ubiquitination is required not only for I κ B degradation, but also for the p100 and p105 precursor processing (fig 2).¹⁶ In addition, proteolysis uncoupled ubiquitination has been shown to be involved in the modification of signalling factors upstream of NF- κ B, such as TRAF6, TAK1/TAB1/TAB2 complexes.¹⁷

I κ B KINASES

The seminal event in the activation of NF- κ B is the phosphorylation of I κ Bs, which is mediated by the IKKs. The 700–900 kDa IKK complex consists of several proteins, three of which are the kinases IKK1 and IKK2 (also called IKK α and IKK β , respectively) and the regulatory subunit NF- κ B essential modulator NEMO (also called IKK γ).¹⁵ The IKK

complex is a converging point for NF- κ B activation by a large number of stimuli. The importance of this complex in NF- κ B activation is further supported by gene targeting analysis. Without the two IKKs or NEMO alone in murine embryonic fibroblast (MEF) cells, NF- κ B activation is completely blocked upon the induction with a variety of stimuli.^{18–19} IKK1 and IKK2 can phosphorylate all three known I κ Bs, namely I κ B α , I κ B β , and I κ B ϵ , in vitro.¹⁵

Although the biochemical functions of IKK1 and IKK2 in vitro appear very similar, genetic analysis has revealed their distinct in vivo functions. Similar to p65, IKK2 mutated mice died from liver apoptosis by day 13–14 after gestation. The IKK2^{-/-} phenotype is rescued by crossing to TNFR1^{-/-} mice, suggesting that the liver apoptosis is likely induced by tumour necrosis factor α (TNF α).²⁰ Lack of IKK2 results in significant decrease in I κ B degradation and NF- κ B activation. Surprisingly, a major defect in IKK1 deficient mice is keratinocyte differentiation, which is independent of both its kinase activity and NF- κ B activity.²¹ In IKK1^{-/-} MEFs, I κ B α phosphorylation and degradation by TNF α and IL-1 is similar to that observed in wild-type MEFs. However, DNA binding activity and induction of certain NF- κ B target genes by TNF α are impaired.^{22–23} This suggests that IKK1 plays a role in enhancing the transactivation function of NF- κ B, which is independent of I κ B degradation. It has been recently demonstrated that IKK1 also functions as I κ B kinase and is essential for I κ B α degradation and NF- κ B activation in RANKL signalling.²⁴ Thus, IKK1 is a critical signalling component in the NF- κ B pathway in response to certain stimuli. Furthermore, IKK1 kinase activity is required for p100 precursor processing induced by NIK, suggesting specificity for substrates besides I κ B α .^{25–26}

NF- κ B ESSENTIAL MODULATOR (NEMO)

The third prominent member of the IKK complex is NEMO, which contains no known intrinsic kinase activity but has the helix–loop–helix and leucine zipper motif known for protein–protein interaction. Mutation in NEMO leads to mouse embryonic lethality due to massive hepatic apoptosis.¹⁹ Cells deficient in NEMO show no NF- κ B activity in response to a variety of stimuli. NEMO is located on the X chromosome and its mutations are associated with two human disorders: incontinentia pigmenti and anhidrotic ectodermal dysplasia with immunodeficiency.^{27–28} Similarly, heterozygous NEMO female mice develop granulocytic infiltration and hyperproliferative keratinocytes with increasing apoptosis.^{29–30} Currently, the mechanism by which NEMO regulates the NF- κ B pathway remains poorly understood. It has been suggested that NEMO activates IKK by recruiting the complexes to the vicinity of other proteins, allowing upstream components to modulate IKK function.³¹ Alternatively, upon interacting with components of upstream signal transduction molecules such as receptor interacting protein (RIP), NEMO undergoes oligomerisation, which activates IKK.³² Enforced oligomerisation of NEMO can activate IKK.³³ Additionally, NEMO may also be subjected to regulation by phosphorylation, for example, mutations of Ser 85 and Ser141 lead to attenuation of phosphorylation of IKK2 and I κ B α in response to TNF α , IL-1 or lipopolysaccharide (LPS).^{34–35}

ELKS

We have identified ELKS as an essential regulatory subunit of the IKK complex. Silencing ELKS expression by RNA interference blocked induced expression of NF- κ B target genes, including the NF- κ B inhibitor, I κ B α , proinflammatory genes such as cyclo-oxygenase 2 and interleukin (IL)-8. These cells were also not protected from apoptosis in response to cytokines. ELKS has functions by recruiting I κ B α to the IKK

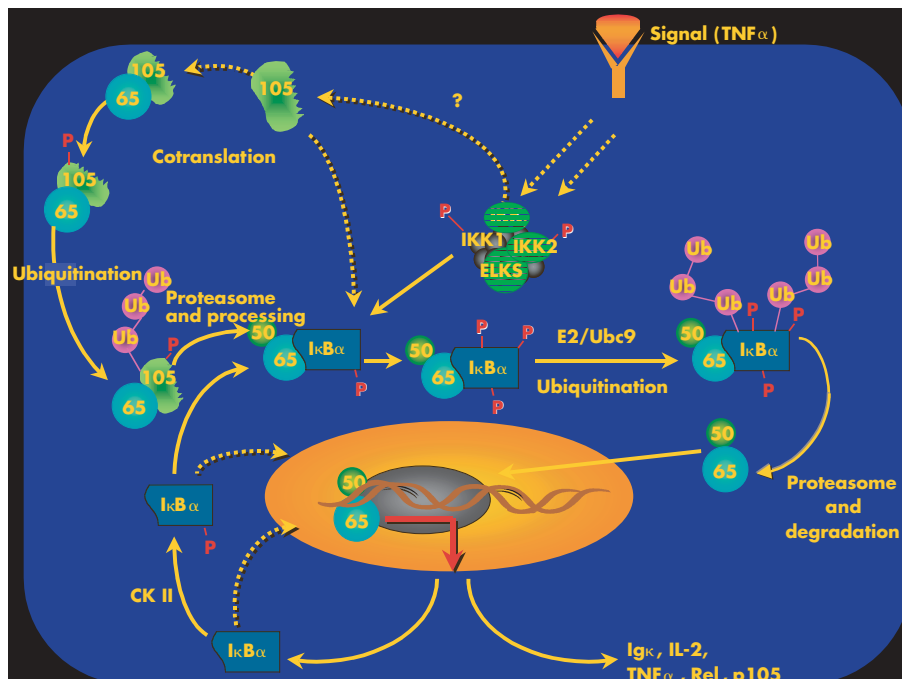


Figure 2 Model for NF- κ B activation. On induction with TNF α , IKK complexes phosphorylate I κ B α , which leads to its degradation and allows NF- κ B dimers to enter the nucleus, where they bind to cognate DNA binding sites and deactivate transcription of genes including I κ B α . I κ B α can then either go to the nucleus and remove the NF- κ B proteins bound to DNA or go to the cytoplasm to prevent further activation. At the same time new p50 is generated by processing of p105.

complex and thus serves a regulatory function for IKK activation. Since ELKS has an essential role in the NF- κ B signal transduction cascade, it may be a suitable target for drug designs aimed at modulating NF- κ B activation for disease intervention.³⁶

ROLE OF THE NF- κ B PATHWAY IN IMMUNE RESPONSES AND INFLAMMATORY DISEASES

NF- κ B is one of the pivotal regulators of proinflammatory gene expression and induces transcription of proinflammatory cytokines, chemokines, adhesion molecules, matrix metalloproteinases, cyclo-oxygenase 2, and inducible nitric oxide (iNOS).^{37,38} NF- κ B is highly activated at sites of inflammation in diverse diseases such as rheumatoid arthritis, inflammatory bowel diseases, multiple sclerosis, psoriasis, and asthma. Electrophoretic mobility shift assays and tissue section staining with specific NF- κ B antibodies consistently detect increased NF- κ B activity with nuclear localisation in biopsies from patients. These changes are accompanied by the enhanced recruitment of inflammatory cells and production of proinflammatory mediators like IL-1, IL-6, IL-8, and TNF α . It is unclear whether increases in proinflammatory cytokine production are the cause or result of NF- κ B activation. Although genetic alterations of NF- κ B and I κ B themselves have not yet been reported to be associated with these diseases, aberrant, constitutive NF- κ B activity could be caused by defects in the regulatory mechanisms controlling NF- κ B activation.

The pathogenic roles of NF- κ B overactivation in inflammatory diseases are supported by studies in p50 and c-Rel knockout mice that are unable to develop eosinophilic airway inflammation when sensitised and challenged with allergen ovalbumin.^{39,40} Specific inhibition of NF- κ B activity has consistently been shown to be effective in controlling inflammatory diseases in several animal models. Blocking NF- κ B activity by overexpression of I κ B α inhibits both the inflammatory response and the tissue destruction in rheumatoid synovium.⁴¹ Administration of NF- κ B decoys appears to be effective in animal models of rheumatoid arthritis.⁴²

NF- κ B AND RESISTANCE TO CHEMOTHERAPY

Chemotherapeutic agents simultaneously induce transcription factors p53 and NF- κ B. p53 induction can activate an apoptotic programme, and resistance to chemotherapy correlates with the loss of a functional p53 pathway. In contrast, NF- κ B prevents apoptosis in response to chemotherapeutic agents. We analysed the p53 response in IKK1/2^{-/-} MEFs, which lack detectable NF- κ B activity. Compared with wild-type fibroblasts, IKK1/2^{-/-} fibroblasts showed increased cell death and p53 induction in response to the chemotherapeutic agent, doxorubicin. Reconstitution of IKK2, but not IKK1, increased Mdm2 levels and decreased doxorubicin induced stabilisation of p53 and cell death. The effects mediated by IKK2 required its kinase function and were abrogated by coexpression of the dominant negative I κ B α M, implying a role for NF- κ B in blocking chemotherapy induced p53 and cell death.⁴³

Our results demonstrate that IKK2, a central regulator of the antiapoptotic pathway controlled by the oncogenic transcription factor NF- κ B, can collaterally downregulate the proapoptotic pathway controlled by the tumour suppressor p53. Our data also distinguish between the functions of the highly homologous kinases, IKK1 and IKK2, in response to chemotherapy. Given that these kinases have distinct *in vivo* roles our results suggest that rather than using a generic IKK inhibitor, specific inhibitors of IKK2 could be used as adjuvants to existing chemotherapy regimens. Collectively, our results provide a mechanism of acquisition of resistance to chemotherapeutic agents that activate both NF- κ B and p53 and also suggest a role for the deregulated NF- κ B activity observed in several leukaemias, lymphomas, and breast cancers.

CLINICAL APPLICATION OF NF- κ B INHIBITORS

Several drugs—ranging from anti-IL-1 and anti-TNF α therapy to widely used anti-inflammatory drugs such as corticosteroids, aspirin, and other non-steroidal anti-inflammatory drugs—used to treat inflammatory diseases have effects on NF- κ B activity.⁴⁴ Although they do not specifically target NF- κ B inhibition, at least some of the effects are due to the inhibition of NF- κ B activation.

Many pharmaceutical companies have programmes to develop selective inhibitors of NF- κ B, which include (a) directly targeting DNA binding activity of individual NF- κ B proteins using small molecules or decoy oligonucleotides; (b) blocking the nuclear translocation of NF- κ B dimers by inhibiting the nuclear import system; (c) stabilising I κ B α protein by developing ubiquitination and proteasome inhibitors; (d) targeting signalling kinases such as IKK using small molecule inhibitors. All these therapeutic strategies are aimed at blocking NF- κ B activity. With increasing knowledge of signalling pathways leading to NF- κ B activation, multiple targets can be identified for potential interaction with small molecules. From the upstream kinases, such as IKK1, IKK2, MEKK-3, and NIK, to their downstream effector I κ B E3 protein,⁴⁵ all represent attractive targets for novel drugs selectively regulating NF- κ B function. Other components of the TNF α and IL-1 signalling pathways including TRADD, RIP, TRAF2, and TRAF6 and IRAK, as well as PKC isoforms and phosphoinositide 3-kinase, may provide additional targets for yet to be discovered inhibitors of NF- κ B.

Although an attractive target for therapeutic intervention in inflammatory diseases, NF- κ B is also involved in normal cellular physiology such as mounting effective immune responses. Global inhibition may result in profound side effects. One of the most prevalent toxicities of NF- κ B inhibition appears to be hepatotoxicity, at least during embryonic development. Even if NF- κ B inhibition is well tolerated by the adult liver, NF- κ B blockade still compromises normal host defence and leaves mice unable to clear opportunistic infections such as that caused by *Listeria monocytogenes*.⁴⁶ Additionally, involvement of NF- κ B in the embryonic development of skin, limb, and bone poses potential dangers. By selectively targeting specific NF- κ B subunits or signalling components involved in a particular disease, one may minimise systemic toxicity. The identification of individual key components for a specific disease is crucial for achieving specific therapeutic aims. It has been shown that IKK2 is important in rheumatoid arthritis synoviocytes, while p65 is associated with inflammatory bowel disease.^{47–48} Furthermore, c-Rel is required for systemic but not local joint disease, while p50 is essential for local joint inflammation and destruction.⁴⁹

FUTURE PERSPECTIVES

The challenge now is to understand how different signal transduction pathways selectively activate different NF- κ B complexes in a coordinated manner. Analysis of the downstream genes affected by NF- κ B is also likely to provide significant insight into the functions of this pathway and could establish connections with other human diseases. In this respect, comparisons between normal mice and NF- κ B mutants with the use of cDNA microarray or DNA chip technology may identify differentially expressed transcripts that are pertinent to NF- κ B function in various physiological and pathological processes. No doubt, better understanding of the NF- κ B signalling pathways involved in specific processes will be beneficial for the development of new generations of anti-inflammatory drugs with high efficacy, fewer side effects, and low cost.

Dr Verma is supported in part by grants from the NIH, the Larry L Hillblom Foundation, Inc, the Lebensfeld Foundation, the Wayne and Gladys Valley Foundation, and the HN and Frances C Berger Foundation.

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