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Review

# New Insights into the Role of Nuclear Factor-κB, a Ubiquitous Transcription Factor in the Initiation of Diseases

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**Nuclear factor-κB (NF-κB) is a ubiquitous transcription factor that governs the expression of genes encoding cytokines, chemokines, growth factors, cell adhesion molecules, and some acute phase proteins in health and in various disease states. NF-κB is activated by several agents, including cytokines, oxidant free radicals, inhaled particles, ultraviolet irradiation, and bacterial or viral products. Inappropriate activation of NF-κB has been linked to inflammatory events associated with autoimmune arthritis, asthma, septic shock, lung fibrosis, glomerulonephritis, atherosclerosis, and AIDS. In contrast, complete and persistent inhibition of NF-κB has been linked directly to apoptosis, inappropriate immune cell development, and delayed cell growth. Therefore, development of modulatory strategies targeting this transcription factor may provide a novel therapeutic tool for the treatment or prevention of various diseases.**

Most human diseases can be ascribed to the aberrant activation and expression of genes whose products are involved in the initiation and progression of pathogenesis *(1–3)*. Such diseases include autoimmune arthritis, glomerulonephritis, asthma, inflammatory bowel disease, septic shock, lung fibrosis, carcinogenesis, and AIDS. In general, these genes are quiescent or have minimal activity in affecting biological and physiological processes. However, under certain conditions that include exposure to environmental pollutants, these genes are abruptly turned on by a preexisting genetic switch, causing their overexpression. Part of this genetic switch is controlled by nuclear factor-κB (NF-κB),[3] an essential transcription factor that controls the gene expression of cytokines, chemokines, growth factors, and cell adhesion molecules as well as some acute phase proteins *(2, 3)*.

NF-κB was first identified as a B-cell nuclear factor and given its name on the basis of its ability to bind to an intronic enhancer of the immunoglobulin κ-light chain gene *(4)*. Since then, NF-κB has been identified in numerous cell types and is found to be activated by a wide range of inducers, including ultraviolet irradiation, cytokines, inhaled occupational particles, and bacterial or viral products. In resting cells, NF-κB resides in the cytoplasm in an inactive form bound to an inhibitory protein known as IκB. Upon cellular activation by extracellular stimuli, IκB is phosphorylated and proteolytically degraded or processed by proteasomes and other proteases. This proteolytic process activates NF-κB, which then translocates into the nucleus. In nuclei, NF-κB can initiate or regulate early-response gene transcription by binding to decameric motifs, "GGGRNNYYCC (κB motif)", found in the promoter or enhancer regions of specific genes.

Although NF-κB binding sites have been identified in the promoter regions of genes whose products are intimately involved in cell-to-cell interaction, it should be emphasized that not all of these genes are up-regulated by NF-κB in a given cell type under every stimulatory condition. Cellular events associated with NF-κB activation include cell-to-cell adhesion *(5–9)*, cell recruitment or transmigration of inflammatory cells *(10, 11)*, amplification or spreading of primary pathogenic signals *(12)*, and initiation or acceleration of tumorigenesis *(13)*. With the onset of simultaneous or asynchronous stimulatory events in any given cell population for a particular

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[3] Nonstandard abbreviations: NF-κB, nuclear factor-κB; IKK, IκB kinase; NIK, NF-κB-inducing kinase; IL, interleukin; CSF, colony-stimulating factor; GM, granulocyte-macrophage; IAP, mammalian inhibitor of apoptosis; Ig, immunoglobulin; AS-ODN, antisense oligonucleotide; ODN, oligonucleotide; and EMSA, electrophoretic mobility shift assay.

stimulus and considering the many transcriptional units involved in gene expression, only a few genes at any one time are affected by NF-κB-mediated transcription. Although NF-κB is thought of as a genetic switch that can control early-response gene expression, the synergistic interaction of NF-κB with other transcription factors such as Stat *(14, 15)*, Ap-1 *(16)*, cAMP-response element binding protein *(17)*, nuclear factor AT *(18, 19)*, and NF-IL6 *(20)* is required to achieve a purposeful induction of a particular gene.

Presently, five mammalian NF-κB family members have been identified and cloned (Table 1). These include NF-κB1 (p50/p105), NF-κB2 (p52/p100), p65 (RelA), RelB, and c-Rel *(1, 2)*. A characteristic feature of NF-κB is that all of the family members share a highly conserved Rel homology domain. This domain is composed of ~300 amino acid residues that are responsible for DNA binding, dimerization, and interactions with IκB, the intracellular inhibitor for NF-κB. The C-terminal regions of RelA, RelB, and c-Rel contain a transactivating domain, which is important for NF-κB-mediated gene transactivation. The C termini of the precursor molecules for p50 and p52, p105 and p100, however, contain multiple copies of the so-called ankyrin repeat, which is found in IκB family members, including IκBα, IκBβ, IκBε, Bcl3, and *Drosophila* cactus. The most abundant activated form of NF-κB is a heterodimer composed of a p50 or p52 subunit and a p65 subunit. Other dimeric complexes, such as p50/p50, p52/p52, RelA/RelA homodimers, and RelA/c-Rel heterodimers, have also been detected in some cell types under certain culture conditions. However, the transactivational properties of these dimeric complexes have yet to be elucidated. Although all of the NF-κB dimers can bind to a common κB binding motif, it has been shown that different dimers recognize slightly different κB motifs. For example, p50/p65 binds the sequence 5′-GGGRNNYYCC-3′, with high affinity, whereas the RelA/c-Rel prefers 5′-HGGARNYYCC-3′ (where H is A, C, or T; R is a purine; and Y is a pyrimidine).

## Kinase Cascade for the Activation of NF-κB

Degradation of IκBα, the inhibitory protein bound to NF-κB, is a key step required for the activation of NF-κB. This process is initiated through signal-induced phosphorylation of two serines (Ser32 and Ser36) on the IκBα molecule *(21–24)*. The phosphorylation event in turn induces polyubiquitination of IκBα on lysines 21 and 22. Phosphorylated and ubiquitinated IκBα can be rapidly recognized and degraded by proteasome, a multiprotease complex *(25)*. Replacement of Ser32 and Ser36 by threonine or alanine residues substantially decreases signal-induced phosphorylation and degradation of the IκBα protein. This suggests the presence of a serine-specific kinase for the phosphorylation of IκBα.

During the past several years, a number of laboratories have investigated the specific serine kinase(s) for IκBα. Although in vitro studies have shown that some known kinases, such as protein kinase C *(26)*, protein kinase A *(27)*, protein kinase R *(28)*, Raf *(29)*, casein kinase II *(30)*, eukaryotic initiation factor-2 kinase *(31)*, and mitogen-activated ribosomal S6 protein kinase *(32)*, are capable of phosphorylating IκBα, none of these kinases is serine-specific or site-specific for IκBα. The first report about a putative IκBα kinase was by Chen et al. *(33)*, who identified a 700-kDa complex that contains kinase activity capable of phosphorylating IκBα at the two crucial serine sites. It is interesting to note that the in vitro activation of this kinase complex requires MAPKKK and/or ubiquitination. At that time, however, no detailed sequence data regarding the structural and molecular characteristics of this IκBα kinase complex were available. In 1997, a major breakthrough in the search for an IκBα-specific kinase occurred with reports by Zandi et al. *(34)*, DiDonato et al. *(35)*, Regnier et al. *(36)*, Woronicz et al. *(37)*, and Mercurio

**Table 1. Mammalian NF-κB family members.**

| Protein | Mass, kDa | Genes | Structure | Knockout phenotype |
|---|---|---|---|---|
| | | | RHD NLS TA ARD PEST | |
| p65 (RelA) | 65 | *rel*A | 551 | Embryonic death |
| RelB | 66 | *rel*B | 582 | Thymic atrophy |
| c-Rel | 68 | c-*rel* | 587 | Reduced cytokine production from T cell and macrophage |
| p50/p105 | 50/105 | *nfkb1* | 969 | Compromised B-cell reaction |
| p52/p100 | 52/100 | *nfkb2* | 940 | Compromised B-cell reaction |
| IκBα | 37 | *med*-3 | 317 | Multifocal inflammation |
| IκBβ | 41 | *ikbb* | 359 | Not determined |
| IκBε | 45 | *ikbe* | 361 | Not determined |
| Bcl3 | 45 | *bcl*3 | 421 | Deficiency in humoral response |

RHD, Rel-homology domain.
NLS, nuclear localization signal.
TA, transactivation domain.
ARD, ankyrin repeat domain.
PEST, proline, glutamic acid, serine, and threonine domain.

et al. *(38)*. Zandi et al. *(34)* and DiDonato et al. *(35)* purified a 900-kDa IκBα kinase (IKK) complex from tumor necrosis factor-α (TNFα)-challenged HeLa cells and cloned an 85-kDa subunit of IKK complex, named IKKα. A gene bank database search demonstrated that IKKα was identical to a putative serine/threonine kinase with unknown function named CHUK (conserved helix-loop-helix ubiquitous kinase). An additional 87-kDa subunit of the IKK complex, IKKβ, was subsequently identified using the same strategy or using a DNA sequence database search. IKKα was also identified independently by Regnier et al. *(36)* and Woronicz et al. *(37)*, who used the NF-κB-inducing kinase (NIK), a MAPKKK family member, as bait in a yeast two-hybrid screen from a human B-cell cDNA library. The polypeptides of IKKα and IKKβ have 52% homology. Both IKKα and IKKβ can phosphorylate Ser32 and Ser36 of the IκBα molecule. A unique structural characteristic of IKKα and IKKβ that differentiates them from other serine/threonine kinases is that both IKKα and IKKβ contain a C-terminal leucine zipper motif and a helix-loop-helix motif. On the basis of the observation that MAPKKK or NIK is required for the in vitro activation of IKK *(33)* and the identification of a canonical MAPKK activation loop motif (SxxxS) on both IKKα and IKKβ *(34–38)*, researchers have speculated that NIK is a direct upstream kinase that can phosphorylate and activate IKKα and IKKβ. Indeed, a coexpression study demonstrated that NIK can phosphorylate IKKα at Ser176, but weakly phosphorylates IKKβ *(39)*. A second potential upstream kinase that activates IKKα and IKKβ is MEKK1, another MAPKKK family member responsible for the activation of Jun-N-terminal kinase and p38. Whereas NIK preferentially activates IKKα, MEKK1 apparently is more potent in the phosphorylation of IKKβ *(40)*. Although NIK has been demonstrated to interact with both IKKα and IKKβ directly *(36)* and MEKK1 has been identified to be a component of the large IKK complex *(38)*, it remains unclear whether other intermediate kinases are involved in the cascade between NIK/MEKK1 and IKKα/IKKβ.

## NF-κB and Cell Apoptosis

The finding that NF-κB is activated during or immediately before cell apoptosis under certain stimulatory conditions has led to the suggestion that this transcription factor may function to promote apoptosis *(41, 42)*. Several lines of evidence support this suggestion. Treatment of human thymocytes and promyelocytic leukemia cells with etoposide activates NF-κB and induces apoptosis *(43)*. NF-κB is concomitantly activated with TNFα-induced apoptosis in certain cell types *(44, 45)*. It has also been shown that inhibition of NF-κB by certain antioxidants prevents apoptosis *(43)*. Indeed, NF-κB binding sites have been identified in the promoters of interleukin-1β converting enzyme protease *(46)*, c-*myc* *(47)*, and TNFα *(48)* genes, which are commonly involved in signal-induced programmed cell death.

The role of NF-κB in the apoptosis process is not straightforward, however. For example, although TNFα and interleukin (IL)-1 are well-known, potent activators for NF-κB, in most cases they do not cause apoptosis unless cells are pretreated with agents that block RNA or protein synthesis *(49)*. In fact, numerous recent studies have clearly demonstrated an antiapoptotic role for NF-κB. Important evidence to support this was provided by the study of p53-independent apoptosis induced by oncogene Ras *(50)*. In Ras-transformed NIH 3T3 cells or p53-deficient ($p53^{-/-}$) mouse embryo fibroblasts, Mayo et al. *(50)* demonstrated that the inhibition of NF-κB by cotransfection of a superrepressor form of IκBα caused a dramatic loss of cell viability. When a similar approach was used, NF-κB was found to be required to overcome cell killing in the human fibrosarcoma cell line HT 1080, in a Jurkat T-cell line, and in human bladder carcinoma induced by TNFα and cancer chemotherapeutic compounds *(51, 52)*. Recently, a NF-κB *rel*A gene knockout mouse model was reported, which has an embryonic lethal phenotype associated with massive liver cell apoptosis *(53)*. This model was created by targeted disruption of the *relA* gene by homologous recombination. Treatment of RelA-deficient ($RelA^{-/-}$) mouse embryonic fibroblasts and macrophages with TNFα led to a substantial reduction in viability, whereas $RelA^{+/+}$ cells from a wild-type mouse were unaffected. Reintroduction of RelA into $RelA^{-/-}$ fibroblasts enhanced cell survival *(54)*. These data suggest that NF-κB is an essential antiapoptotic factor for these cell types.

Additional evidence that NF-κB inhibits apoptosis was obtained from the study of anti-IgM-induced apoptosis in WEHI 231 immature B-lymphoma cells. After treatment of these cells with L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone or microinjection of glutathione-*S*-transferase-IκBα, the protective role of NF-κB against anti-IgM-induced cell apoptosis was eliminated *(55)*. Furthermore, NF-κB was found to be protective in HIV-Tat and exogenous nitric oxide (NO)-induced apoptosis (Chen et al., unpublished data).

How can NF-κB mediate both life and death signals in cells? One possibility is that different NF-κB members mediate different signals. Evidence in favor of this possibility comes from the studies of NF-κB gene knockout mice. For example, *relA* gene disruption caused embryonic death in mice *(53)*. However, no developmental abnormalities were found in p50 gene knockout mice *(56, 57)*. A second possibility is that the role of NF-κB in apoptosis depends on the cell type or the type of stimulation that determines which simultaneous and asynchronous signaling pathway are activated. Evidence to support this possibility comes from the studies of oncogenic Ras-transformed cells and Bcr-Abl-transformed cells. Whereas NF-κB is clearly protective for the cells against Ras-induced apoptosis *(50)*, it is not required in the protection of apoptosis induced by IL-3 withdrawal or

exposure to etoposide and ionizing radiation in Bcr-Abl-transfected cells *(58)*.

The protective role of NF-κB against apoptosis may occur through the up-regulation of genes encoding anti-apoptotic products such as IL-1, IL-2, IL-6, macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), superoxide dismutase, and the zinc finger protein A20 *(1–3)*. The regulation of NF-κB on other antiapoptotic genes remains to be further elucidated. For example, the expression of a newly cloned antiapoptotic gene, *bcl*-x, may be involved as a downstream protective gene of NF-κB. In this regard, we recently characterized several κB-like elements in the 5′-flanking region of the mouse *bcl*-x gene (Chen et al., unpublished data). These κB-like elements may be involved in the regulation of the *bcl*-x gene. Additional genes implicated include the genes that code for mammalian inhibitor of apoptosis (IAP), where both c-IAP1 and c-IAP2 have been shown to be up-regulated by NF-κB. In human blood T cells and a Jurkat T-cell line, the expression of c-IAP2 is under the control of NF-κB *(59)*. Furthermore, the accumulation of c-IAP2 mRNA induced by TNFα can be substantially attenuated by the inhibition of NF-κB either by transfection of cells with degradation-resistant IκBα or by treating the cells with a proteasome inhibitor. Changes in NF-κB, however, had little effect on the expression of c-IAP1, which is in disagreement with the observation reported by You et al. *(60)*, who showed that c-IAP1 was highly expressed in chicken fibroblasts transfected with NF-κB v-*rel*. The involvement of cIAPs in NF-κB-mediated antiapoptosis was further demonstrated by the studies of intracellular expression of cIAPs. In the HT1080 fibrosarcoma cell line, in which NF-κB was inactive, expression of cIAPs was sufficient to suppress etoposide-induced apoptosis *(61)*. Because of the lack of detailed genomic DNA sequence data for either mammalian c-IAP1 or c-IAP2, one cannot determine at this time if the dependence of c-IAP1 and c-IAP2 on NF-κB is through the κB or κB-like site(s) located in the promoter or enhancer regions of the c-IAP1 and c-IAP2 genes.

## NF-κB in Development

When NF-κB was first named by Sen and Baltimore *(4)* on the basis of its binding to the enhancer of the immunoglobulin (Ig) κ gene in B cells, it was speculated that NF-κB acts as a key transcription factor for the development of the immune system, especially for B cells *(4)*. Indeed, recent gene knockout studies provided convincing evidence that a deficiency in B cells occurred when the genes *nfkb1* (p50/p105), *nfkb2* (p52/p100), and c-*rel* were disrupted. B cells from *nfkb1* knockout mice were virtually unresponsive to lipopolysaccharide stimulation when compared with B cells from control mice *(56, 57)*. In addition, the total Ig production and certain kinds of germ-line Ig type switching were markedly impaired in B cells from *nfkb1* knockout mice. In contrast, the Ig type switching was unaffected in *nfkb2* knockout mice, although the absolute number of B cells in peripheral lymphoid organs decreased and the B cells exhibited a reduced lipopolysaccharide response *(62, 63)*. In c-*rel* gene knockout mice, development of cells from all hemopoietic lineages appeared normal, although both mature B and T cells were unresponsive to most mitogenic stimuli *(64)*.

It is becoming more and more evident that NF-κB is a key factor not only for the development and function of B cells, but also for the development and function of many other cells, including T cells, thymocytes, dendritic cells, macrophages, and fibroblasts. Targeted disruption of the *relB* gene caused varying degrees of thymic gland atrophy and lymphoid cell depletion in lymph nodes *(65, 66)*. In addition, T-cell-mediated immune response and dendritic cell development were strongly impaired. A double knockout of *nfkb1* and *nfkb2* demonstrated an unexpected phenotype. Mice developed osteopetrosis or Albers-Schoenberg disease as a result of diminished bone resorption caused by a deficiency in osteoclast lineage development *(67, 68)*. Additional studies suggested that this deficiency occurred as a result of a defect either during osteoclast precursor cell differentiation or during the maturation of osteoclasts. Obviously, this double knockout experiment could provide a relevant model to study the etiology of osteoporosis and to develop new therapeutic strategies for the treatment of this disease. It has been shown that several cytokines, including TNFα, IL-1, IL-6, and GM-CSF, have osteoclastogenic activity and are increased in osteoblasts and hematopoietic cells of patients with decreased estrogen concentrations *(67)*. There is also some evidence to show that occupied estrogen receptors inhibit IL-6 gene expression by preventing the binding of NF-κB and NF-IL6 transcription factors to their respective binding sites in the IL-6 gene promoter *(69)*. A similar mechanism may also be involved in the inhibition of TNFα and IL-1 production by estrogen.

NF-κB may also play a critical role in embryonic development. Well-documented evidence from the studies of *Drosophila* embryogenesis support this role of NF-κB. Dorsal, a fly homolog of NF-κB family members, is vital for the establishment of the embryonic dorso-ventral axis during development *(70)*. Similarly, in a mouse gene knockout study, disruption of the *relA* locus led to embryonic death at 15–16 days of gestation, accompanied by massive degeneration of the liver as a result of apoptosis *(53)*. Two separate groups recently provided additional evidence to show that NF-κB is required for the development of vertebrate embryonic limbs *(71, 72)*. They found that NF-κB genes were expressed in the progress zone of the developing chick limb bud and were maintained until the last stage. Inhibition of NF-κB activity by infection with viral vectors that produce transdominant-negative IκBα protein caused a highly dysmorphic apical ectodermal ridge, reduction in overall limb size, loss of distal elements, and arrest of budding.

The exact role of NF-κB family members in the development of cell lineages and embryonic development remains to be defined. It should be possible in the near future to answer the specific, key questions that relate to these issues. For example, which member or combination of members of the NF-κB family is essential for a specific developmental stage or which gene or gene set is regulated by NF-κB for the differentiation and maturation of a given cell lineage.

## NF-κB in Carcinogenesis

Evidence for a potential role for NF-κB in carcinogenesis is provided by the observation that activation of NF-κB is required in oncogenic Ras-induced transformation *(50)*. Upon inhibition of NF-κB activation with a superrepressor form of IκBα, oncogenic Ras-transformed cells exhibit a loss of cell viability, indicating that oncogenic Ras requires the cell survival function of NF-κB to overcome the role of the death signal initiated in transformed cells. Similarly, NF-κB is required for leukemogenesis initiated by Bcr-Abl chimeric protein (a deregulated tyrosine kinase) *(58)*. The alternative activation or expression of NF-κB is evident in several human cancers, including breast cancer *(73, 74)*, non-small cell lung carcinoma *(75)*, thyroid cancer *(76)*, T- or B-cell lymphocyte leukemia *(77)*, and several virally-induced tumors *(78–81)*. Thus, a role for NF-κB in the malignant transformation of cells is highly possible. Nevertheless, it is unclear whether overactivation or excessive expression of NF-κB in these transformed cells is linked directly to the transformation or whether NF-κB only provides an accessory signal for the transformation.

Using an estrogen receptor-negative breast cancer cell lines, Sovak et al. *(73)* and Nakshatri et al. *(74)* showed that NF-κB was continuously activated and could be correlated with poor differentiation and high metastasis of these cancer cells. Aberrant NF-κB activation has also been observed in carcinogen-induced primary rat mammary tumors and multiple human breast cancer specimens. In human lung cancer, NF-κB p50 and c-Rel were found highly expressed in fresh human non-small cell lung carcinoma tissues and cell lines *(75)*. In contrast, the expression of NF-κB p52 was very low or undetectable in these tumors or cell lines. On the other hand, the constitutive nuclear localization of NF-κB p50/p65, the most abundant heterodimer in signal-induced cells, was found to be required for the sustained proliferation of Hodgkin lymphoma and was considered to be a potential diagnostic marker for this disease *(77)*.

Chromosomal alterations of the NF-κB or IκB gene family have been noted frequently in several human lymphoid tumors in which rearrangement of the *nfkb2* gene is described more commonly *(82)*. Rearrangement of the *nfkb2* gene causes deletions of sequences encoding the ankyrin repeat motif of p100. Consequently, this carboxy-terminal truncated p100 is constitutively located in the nucleus of cells. Similarly, some groups have identified rearrangement of the c-*rel* gene in numerous non-Hodgkin lymphomas *(83)*. The *bcl*3 gene, which encodes an IκB-like protein that can regulate transcriptional activity of NF-κB p50 or p52 homodimer, is rearranged at a common chromosomal breakpoint of chromosome 19q13.1 in most cases of chronic lymphocytic leukemia with t(14:19) translocation *(84)*. Unlike rearrangement of the *nfkb2* gene, alterations at the *bcl*3 locus do not truncate or change the coding sequence, but rather cause overexpression of *bcl*3 mRNA.

It is well known that some human tumors result from viral infections. These tumors include HTLV-1-induced acute leukemia of $CD4^+$ T cells *(78)*, Epstein-Barr virus-induced Burkitts and Hodgkin lymphoma *(79)*, hepatitis B virus-induced hepatocellular carcinoma *(80)*, and HIV-induced Kaposi sarcoma *(81)*. NF-κB can be activated rapidly by Tax *(85)*, latent membrane protein-1 *(86)*, X protein *(87)*, and Tat protein *(88)* encoded by HTLV-1, Epstein-Barr virus, hepatitis B virus, and HIV, respectively. Therefore, NF-κB is thought to be a common mediator for viral-induced tumorigenesis. Convincing evidence to support this hypothesis is provided by the observation that mice treated with antisense oligonucleotides (AS-ODNs) to *rel*A (NF-κB p65) have a reduced incidence of Tax-induced tumors *(89)*.

The most direct evidence for a role for NF-κB in oncogenic transformation has been derived from the fact that v-Rel, a member of the Rel/NF-κB family of eukaryotic transcription factors, induces oncogenic transformation in avian lymphoid cells *(76)*. Recent studies demonstrated that v-Rel also has the capacity of transforming mammalian cells in vivo *(90)*. Transgenic mice expressing v-Rel under the control of the *lck* T-cell-specific promoter develop T-cell lymphomas. There are two major DNA-binding complexes found in tumor cells from v-Rel-transgenic mice, one containing v-Rel homodimers and another containing v-Rel/p50 heterodimers. Additional evidence that supports an oncogenic role of v-Rel in mammalian cells has been obtained from the study of overexpression of the Rel/NF-κB inhibitor protein, IκBα, in v-Rel transgenic mice. Overexpression of IκBα prolongs the survival of v-Rel transgenic mice and delays the development of T-cell lymphomas in this mouse model *(91)*. Furthermore, a functional role for v-Rel in transformation is also indicated by the observations that a v-Rel mutant that cannot form homodimers cannot transform cells and that the addition of a nuclear export signal to v-Rel blocks its transforming ability *(92, 93)*. Together, these data provide strong support for the hypothesis that v-Rel contributes to direct malignant transformation of eukaryotic cells.

The target genes influenced by NF-κB in carcinogenesis have yet to be defined. The fact that NF-κB regulates genes for both cell carcinogenesis and apoptosis has led to substantial confusion concerning the role for NF-κB on pretransformation or antitransformation. Additional studies

are clearly required to unveil the molecular basis for the activation and function of NF-κB in transformation.

### NF-κB as a Therapeutic Target for Diseases

The key role that NF-κB plays in controlling the expression of multiple inflammatory and immune genes involved in toxic shock, acute phase responses, radiation damage, asthma, rheumatoid arthritis, atherosclerosis, cancer, and AIDS makes this factor a central and favorable target for therapeutic intervention of diseases *(1–3)*. Most of the biological and biochemical inhibitors of NF-κB presently available act by blocking the signaling pathways that lead to the activation of NF-κB or by compromising the binding activity of NF-κB to target DNA.

The signaling pathway in which IκB is phosphorylated, ubiquitinated, and subsequently degraded by proteasome or other proteases provides numerous potential target points for interference. Some of these points may serve as relatively specific targets, such as the TNF/IL-1 receptor, receptor-associating proteins, or IKK complex, whereas others may be less specific, such as interference with ubiquitin-conjugating enzymes and proteasome (Fig. 1). Many antioxidants inhibit NF-κB by blocking upstream signaling that leads to phosphorylation of IκB. Antioxidants can potentially prevent oxidation of redox-sensitive cysteines in kinases or phosphatases *(94)*. There is evidence to show that proteasome and calpain inhibitors such as MG132, lactacystin, and calpastatin are potent inhibitors of NF-κB activation. They act by blocking the degradation of IκB *(95, 96)*. However, it is important to recognize that both proteasome and calpain are important

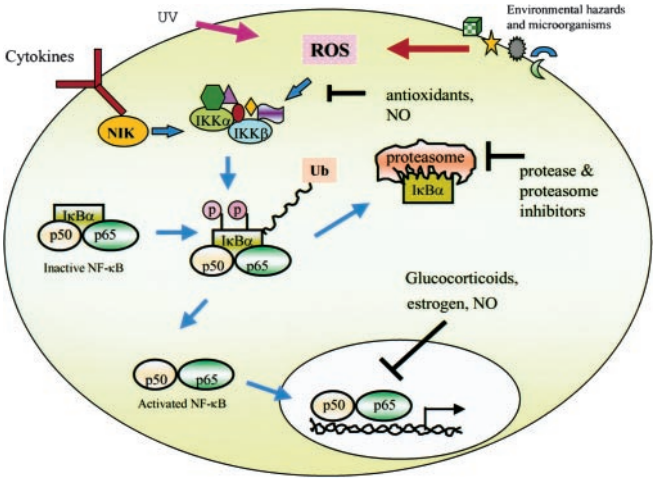


Fig. 1. NF-κB activation and its inhibition.

In response to extracellular inducers, IκB kinase (*IKK*) is activated and can phosphorylate IκBα associated with NF-κB p50/p65 heterodimer. The activation of IKK and phosphorylation of IκBα can be blocked by antioxidants and NO. Phosphorylated IκBα then serves as a substrate for ubiquitination, which is followed by degradation of IκBα by proteasomes. The inhibitors of proteasomes or proteases can block this process. After degradation of IκBα, the p50/p65 complex translocates into the nucleus and binds to the κB-sites of gene promoters. Both glucocorticoids and NO can decrease the DNA-binding activity of NF-κB. *ROS*, reactive oxygen species; *Ub*, ubiquitin; *UV*, ultraviolet light; →, activation; ⊢, inhibition.

regulators for normal cellular function and cell cycling as well. Therefore, the clinical application of general proteasome or calpain inhibitors would be questionable. Glucocorticoids are well-known and widely prescribed immunosuppressive and antiinflammatory drugs. Recent studies have shown that the antiinflammatory effects of glucocorticoids are achieved either by occupied glucocorticoid receptor-mediated interference of NF-κB DNA-binding activity or by enhanced synthesis of IκBα, which would compromise the nuclear translocation and DNA binding of NF-κB *(97–99)*. Thus, glucocorticoid therapy may down-regulate the activation of NF-κB in select circumstances. The liberal use of glucocorticoids, however, is limited because of their well-known side effects on endocrine function and metabolism.

NO-generating compounds such as nitroglycerin and nitrofurantoin have been used for decades for the treatment of cardiovascular diseases *(100)*. NO is a ubiquitous free radical that promotes vasodilation and other intracellular and intercellular biological events. Most radicals are potent activators of NF-κB. However, this is not the case for NO. We have shown that the endogenous induction of NO inhibits NF-κB *(101, 102)*. We assumed that this inhibition is because of direct S-nitrosylation of NF-κB. This assumption was confirmed recently by Matthews et al. *(103)*, using electrospray ionization mass spectrometry in which they found that cysteine 62 *(59)* of p50 was nitrosylated by NO and that the nitrosylation of cysteine decreased the NF-κB DNA-binding activity. In addition to its direct modification of NF-κB, NO is also capable of interfering with several signaling pathways that lead to the activation of NF-κB. In fact, in human endothelial cells, Spiecker et al. *(104)*, Shin et al. *(105)*, De Caterina et al. *(106)*, and Peng et al. *(107)* observed that inhibition of NF-κB by NO is through the stabilization of IκBα and/or the enhancement of IκBα synthesis. Our most recent studies with macrophages and human T cells have shown that both endogenous and exogenous NO can retard signal-induced IκBα degradation by suppressing proteasome activity (Chen et al., unpublished data). It is also possible that NO functions as a $H_2O_2$ scavenger and antioxidant to inhibit activation of NF-κB by other free radicals *(95)*. Therefore, the benefit of NO on cardiovascular diseases is not only through its vascular relaxation properties, but also by its ability to decrease NF-κB-mediated IL-1, TNF, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 expression. These effects can lead to a reduced risk of atherosclerosis.

Transdominant IκBα mutants have been widely used in various experimental systems. The IκBα mutated at Ser32/Ser36 or Lys21/Lys22 is much more resistant to ubiquitination and degradation by the proteasome, but it is still able to associate with and sequester NF-κB. Specific inhibition of NF-κB by transfection and expression of this IκBα mutant has been achieved in studies of oncogenic Ras and Bcr-Abl-induced transformation and apoptosis and numerous other experiments *(50, 51, 58)*. Yet the

therapeutic use of this approach remains to be defined. A more rational and promising strategy to block NF-κB activation and its role in disease initiation is the development of oligonucleotide-based interventions. From a therapeutic perspective, this approach potentially targets a specific point in the signaling pathway to inhibit NF-κB without influencing other cellular biological functions. There are two types of oligonucleotides presently being tested. One type is an antisense oligonucleotide (AS-ODN) that binds to a selected mRNA by Watson-Crick base pairing to ablate translation of selected gene products. Compelling evidence for the usefulness of this approach has been obtained from an experiment involving 2,4,6-trinitrobenzene sulfonic acid-induced inflammatory colitis *(108)*. Local administration of p65 antisense phosphorothioate oligonucleotides abrogated clinical and histological signs of trinitrobenzene sulfonic acid-induced colitis. Similarly, treatment of rheumatoid synoviocytes or fibroblasts with p65 AS-ODN decreased IL-1β-induced cyclooxygenase-2 protein expression and prostaglandin $E_2$ production *(109, 110)*. A second type of oligonucleotide used to intervene in the function of NF-κB is a decoy of double-stranded ODNs containing a NF-κB-binding element. This decoy acts as a competitor to block the binding of NF-κB to promoter regions of target genes, thus inhibiting gene transactivation. The first evidence for the potential of this NF-κB decoy was obtained from the study of HIV-LTR transactivation in Epstein-Barr virus-transformed B cells. Treatment of cells with NF-κB decoy DNA strongly inhibited HIV-LTR activity *(111)*. Recently, this decoy strategy was used successfully in studies of NF-κB-mediated lymphocyte cytokine production, IgE isotype switching, and ischemia-reperfusion myocardial infarction *(112–114)*. The reports describing the use of NF-κB AS-ODNs or decoys are very promising. However, a wide variety of unexpected sequence-independent effects have come to light and consequently compromise rational drug design and anticipated single gene elimination.

### Determination of NF-κB Activation and Its Function

Although much research needs to be done, it is highly possible that NF-κB may be used as a diagnostic index to guide therapeutic strategies for certain diseases. Several methods to determine NF-κB activation or its function have been widely used in research laboratories, such as the electrophoretic mobility shift assay (EMSA), in situ hybridization, Western blot, and the reporter gene assay. Because NF-κB is translocated into nuclei after the degradation of its inhibitor, IκBα, the amount of NF-κB proteins in nuclear extracts may authentically reflect the activation status of NF-κB. EMSA is a traditional and simple method used to determine the inducible and constitutive NF-κB in nuclei. To do this, a total nuclear extract is incubated with a α-$^{32}$P-labeled double-stranded ODN containing a consensus κB-site, usually GGGAATTCCC. The protein bound to the κB probe can be resolved by 5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. If specific antibodies are included during the incubation of nuclear extract with the κB probe, the member composition of the NF-κB complex can also be identified using this approach.

In combination with histopathological and cytological studies of tumor tissue and inflammatory tissue, nuclear translocation of NF-κB can also be quantified by use of fluorescence-labeled antibodies against different NF-κB family members in tissue sections. There are several major advantages of this approach to determine nuclear translocation of NF-κB. First, the tissue or cell morphology is well preserved, and it provides precise localization of various NF-κB members. Second, use of this method avoids the problem of radioactive materials and false stress signals, which may happen when nuclear extracts and $^{32}$P-labeled probes are used in EMSA. Third, one can accurately identify the population of cells expressing NF-κB members.

For cell suspension samples such as blood cells, both patch clamp and flow cytometric methods have been used to determine the translocation of NF-κB through nuclear pore complex and DNA binding, respectively. During nuclear translocation, NF-κB may plug the nuclear pore complex channel and thus interrupt ion flow through the channel. The reduction in the ion conductance of the channel can be recorded by patch clamp detection *(115)*. By the use of a flow cytometer equipped with double optical filters that allow detection of propidium iodide-stained nuclei and fluorescein isothiocyanate-stained NF-κB, the translocation of NF-κB into nuclei can also be identified in mononuclear cells and neutrophils without preseparation of cell populations *(116)*. However, the requirement for technical expertise and specific equipment compromises the potential application of these two methods in clinical laboratories.

The functional characteristic of NF-κB can be determined experimentally by assaying the expressing κB-dependent reporter genes. Traditionally, a reporter construct containing chloramphenicol acetyltransferase or luciferase reporter genes under the control of κB elements is transfected into cells. Upon stimulation, the reporter gene activity in cells can be detected by ELISA, thin-layer chromatography, a luminometer, or a scintillation counter. For clinical purposes, the transactivational activity of NF-κB can be measured indirectly by determining the production of cytokines, cell adhesion molecules, and enzymes whose genes are dependent on NF-κB transcription factor.

### Conclusion

Regulation and control of NF-κB activation can be a powerful therapeutic strategy for reducing tissue damage as a consequence of the releases of inflammatory mediators. In addition, controlled regulation of NF-κB activation has the potential to increase the sensitivity of tumor cells to antitumor therapy. However, a complete and persistent blockage of NF-κB activation will lead to im-

mune deficiencies and the apoptosis of healthy cells. Studies are underway to develop NF-κB member-specific and cell type-specific drugs that can inhibit the activation of NF-κB only in target cells. Because of the key role that this factor plays in the expression of proinflammatory genes and its importance to the function of the immune system, intense research is presently being carried out to further delineate the role and function of this important nuclear factor in health and pathogenesis.

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