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REVIEW

Rel/NF-κB/IκB family: intimate tales of association and dissociation

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The growth, differentiation, and development of an organism is orchestrated by the choreographed expression of a wide array of genes. The switching "on" and "off" of gene expression is the province of transcription factors, which operate singly or in association with other proteins. Usually transcription factors form families, whereas individual members perform specific, distinct, or similar tasks. One such family includes the Rel/NF-κB proteins (NF-κB), which have the unique property of being sequestered in the cytoplasm in association with inhibitory proteins called IκB.

Activation and regulation of NF-κB are tightly controlled by IκB proteins. Through noncovalent association, the IκB proteins mask the nuclear localization signal (NLS) of NF-κB, thereby preventing NF-κB nuclear translocation. Upon stimulation with signaling molecules such as tumor necrosis factor α (TNFα) or lipopolysaccharide (LPS), NF-κB is released from IκB and translocated to the nucleus where it regulates gene transcription. Stimulation and activation of the NF-κB transcription factors do not require protein synthesis, thereby allowing rapid and efficient activation of target genes. This system is particularly utilized in immune, inflammatory, and acute phase responses where rapid activation of defense genes following exposure to pathogens such as bacteria and viruses is critical for survival of an organism. Many pathogenic viruses have evolved enhanced viral replication by including NF-κB target sites in their promoter/enhancer elements and producing proteins that activate NF-κB. Besides the immune responses and viral replication, NF-κB is also implicated in cellular proliferation and programmed cell death. Coincidentally, deregulation of NF-κB activity is directly associated with cellular transformation. The NF-κB homologs, Dorsal and Dif in the fruit fly *Drosophila*, are involved in the formation of embryonic polarity and insect immunity, respectively. Thus, the understanding of the regulation of Rel/NF-κB activity is of great interest to a wide variety of basic biological and medical fields. Current data lend credence to the notion that the major regulatory step for NF-κB activation is post-translational modifications of the NF-κB inhibitor IκB. In this review we summarize recent studies on NF-κB regulation, with particular emphasis on IκB modification. For earlier studies readers are encouraged to turn to many previous reviews (Baeuerle 1991; Nolan and Baltimore 1992; Baeuerle and Henkel 1994; Beg and Baldwin 1993; Siebenlist et al. 1994; Israel 1995; Kopp and Ghosh 1995; Miyamoto and Verma 1995; Thanos and Maniatis 1995).

Rel/NF-κB family of transcription factors

NF-κB was originally discovered as a lymphoid specific protein that bound to the decameric oligonucleotide GGGACTTCC, present in the intronic enhancer element of the immunoglobulin κ light chain (Igκ) gene (Sen and Baltimore 1986). Purification and subsequent cloning of genes encoding dimeric NF-κB subunits p50 (NFκB1) and p65 (RelA) revealed a surprising homology to the oncogene v-*rel* and the *Drosophila* morphogen *dorsal* (Stephens et al. 1983; Steward 1987; Bours et al. 1990; Ghosh et al. 1990; Kieran et al. 1990; Meyer et al. 1991; Nolan et al. 1991; Ruben et al. 1991). Other members of this protein family include NFκB2 (p52) and RelB (Bours et al. 1992; Mercurio et al. 1992; Ryseck et al. 1992). The Rel/NF-κB family of proteins share an amino-terminal ~300-amino-acid domain (Rel homology domain, RHD), including DNA-binding and dimerization domains and the nuclear translocation signal (NLS), which is most likely the binding site for IκB (Fig. 1; Miyamoto and Verma 1995). Most members can form homo- and heterodimers in vitro except for RelB, which only forms dimers with p50 or p52 (Dobrzanski et al. 1993, 1994; Ryseck et al. 1992; Bours et al. 1992). Another proposed NF-κB family member, NF-AT (nuclear factor of activated T cells), has limited sequence homology (Nolan 1994); however, NF-AT is not found in dimers with other Rel/NF-κB members nor does it bind to Rel/NF-κB DNA-binding sites.

The DNA-binding and dimerization domains in the RHD are distinct from other well characterized motifs. Their structures remained elusive despite many attempts to define them by mutagenesis of the RHD. Structural assignment for these functional domains was,

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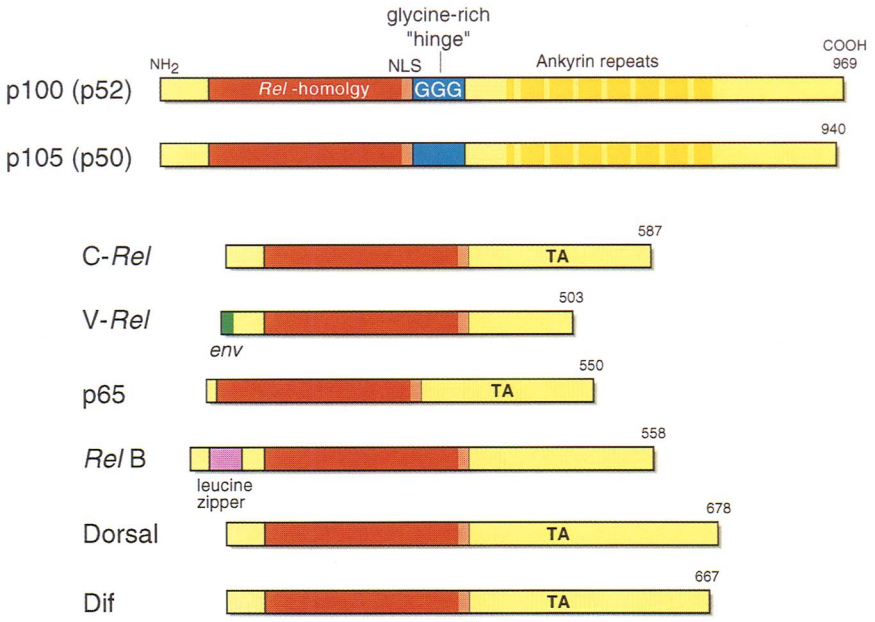

Figure 1. The Rel/NF-κB/Dorsal family. All the members of this family contain the well-conserved Rel homology region (red box) which is ~300 amino acids long and is involved in DNA binding and dimerization. The NLS (pink box), a stretch of basic amino acids, is also well conserved. (TA) *Trans*-activation domain present in c-Rel, p65, Dorsal, and Dif. Both p50 and p52 are generated by a proteolytic processing event of the precursor p105 and p100 possibly involving the glycine "hinge" (blue box) region. The carboxy-terminal half of p100 and p105 contains the anykyin repeat motif (orange boxes).

however, recently accomplished by X-ray crystallographic analyses of bacterially expressed p50 homodimer bound to its cognate DNA-binding sites (Fig. 2) (Ghosh et al. 1995; Muller et al. 1995). The DNA base recognition is unusual and is mediated by peptide loops entering major grooves of the target DNA. In addition to DNA bases, many backbone phosphate groups are also involved in stabilization of the protein–DNA complex of an unusually high affinity with a dissociation constant of $\sim 10^{12}$ M (see review by Baeuerle 1991). The base and phosphate groups of the DNA consensus sequence 5′-GGGRNNYYCC-3′ (κB site) are recognized by conserved residues throughout the RHD of p50. In contrast, p50 dimerization is exclusively mediated by a ~100-amino-acid domain in the carboxyl terminus of the RHD and is stabilized by a series of β-sheets involving core hydrophobic and peripheral hydrophilic residues.

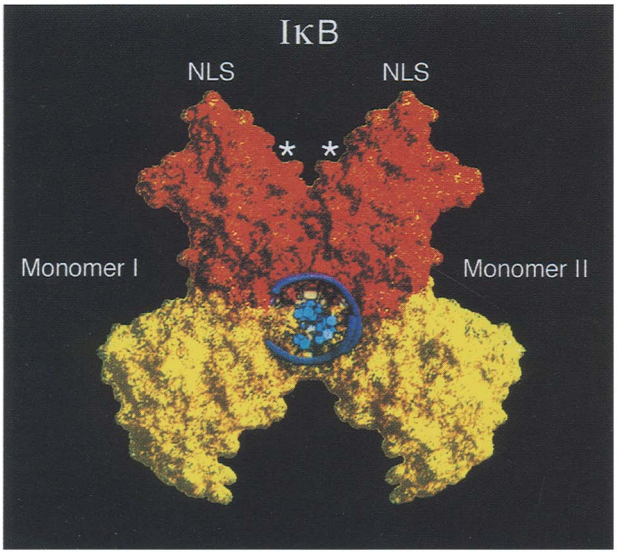

Figure 2. Model of p50 dimer bound to DNA. This model shows the surface of the NF-κB p50 dimer with the dimerization domain (red) and the engulfed DNA (blue). The cleft between the dimerization surface could be the site of IκB docking (shown by an asterisk), thereby blocking the NLS. Reproduced, with permission, from Müller et al. (1995).

The majority of amino acid residues involved in DNA and dimer contacts are conserved in Rel/NF-κB family of proteins; therefore, the specific DNA-binding and dimer partner preferences must be determined by subtle amino acid differences in the DNA-binding and dimerization domains. Some of the critical residues for specific DNA binding lie in the amino-terminal "DNA recognition loop" (Muller et al. 1995), because as little as a 4-amino-acid difference in this region between p50 and p65 determines the DNA recognition specificity of each protein (Coleman et al. 1993). Similarly, subtle differences in the dimerization domain also play a critical role in determining dimer partner specificity (Ruben et al. 1992; Ganchi et al. 1993). In this light, it is of interest to determine what unique residues of RelB allow heterodimerization with p50 and p52 while discriminating homodimerization or heterodimerization with c-Rel and p65. Additional crystallographic analyses of different Rel/NF-κB dimers on κB sits will surely help in defining the different DNA recognition specificity generated by each dimer and dimer partner preferences.

p50 and p52 do not generally activate transcription as homodimers because they lack a potent transcriptional activation domain (Fig. 1). In addition, they are synthesized as cytoplasmic precursors p105 and p100, which perform IκB functions in preventing them from binding

to κB sites (Bours et al. 1990, 1992; Ghosh and Baltimore 1990; Kieran et al. 1990; Meyer et al. 1991; Neri et al. 1991; Rice et al. 1992; Schmid et al. 1991; Mercurio et al. 1992, 1993; Henkel et al. 1993). Proteolytic generation of p50 and p52 from their precursors is signal dependent and thus a regulatory step that generates transcription factors (Rice et al. 1992; Mercurio et al. 1993). The processing of p105 to p50 requires ATP and is mediated by a ubiquitin-dependent proteasome degradation pathway (Palombella et al. 1994). Production of a transcription factor from a non-nuclear precursor protein by proteolysis is also observed for SREBP-1 (sterol response element binding protein) where the precursor bound to the endoplasmic reticulum is cleaved in response to a reduction of the level of sterol in the cellular environment (Wang et al. 1994). In addition to p105 and p50, NFκB1 also generates an IκB member, IκBγ, from an internal intronic promoter (Inoue et al. 1992; J. Inoue, pers. comm.). Furthermore, alternative splicing of RNA transcripts encoded by the murine p105 NF-κB1 gene generates IκBγ isoforms with distinct inhibitory activities (Grumont and Gerondakis 1994a). One spliced transcript encodes a nuclear isoform of the p50 precursor of NF-κB1 that can function as a *trans*-activator of NF-κB regulated transcription (Grumont et al. 1994). No IκBγ-like inhibitor encoded by the NFκB2 gene (p100/p52) has yet been identified.

In contrast to p50 and p52, other members of the Rel/NF-κB family (p65, c-Rel, RelB, Dorsal, Dif) are not generated from precursor proteins and possess a carboxy-terminal *trans*-activation domain (for review, see Miyamoto and Verma 1995). Thus, Rel/NF-κB dimers could act as a transcriptional activators or repressors at κB sites, depending on whether members in a dimer contain or lack a *trans*-activation domain. The κB sites are present in the regulatory regions of genes involved in immune response (Igκ, IL-2, and IL-2Ra), inflammatory, and acute phase responses (IL-1, IL-6, TNFα, TNFβ, and serum amyloid A protein), viruses (HIV-LTR, SV40, CMV, and adenovirus), Rel/NF-κB members (c-Rel, NFκB1, NFκB2, and RelB), IκB members (IκBα, IκBγ, p105, p100, and Bcl-3), growth control proteins (p53, c-Myc, Ras), and cell adhesion molecules (I-CAM, V-CAM, and E-selectin), as well as many other genes (for review, see Baeuerle 1991; Grilli et al. 1993; Collins et al. 1995; Kopp and Ghosh 1995; Miyamoto and Verma 1995). The nucleotide sequence of κB sites can dictate the affinity of Rel/NF-κB dimer interaction. Additionally, some κB sites are known to cause conformational changes that are correlated with transcriptional activation by p50 homodimers in vitro (Fujita et al. 1993). Conversely, NF-κB induces DNA bending in κB sites and does not bind to methylated κB sites (Schreck et al. 1990). Furthermore, NF-κB *trans*-activation activity may be augmented or diminished by the factors that bind to other *cis*-acting elements present in the proximity of κB sites (for review, see Miyamoto and Verma 1995). Therefore, the activity of Rel/NF-κB dimers can be modulated directly by the κB sites and nearby DNA sequences and other DNA-binding proteins.

The finding that oncoprotein v-Rel belongs to the Rel/NF-κB family suggested that other family members may also play roles in cellular transformation. The increased activity of NF-κB has been implicated in the proliferation of murine fibroblasts transformed by the Tax protein encoded by the human T-cell leukemia virus-1 (Kitajima et al. 1992). Additionally, antisense oligonucleotides for *relA* (p65) mRNA reduced cell adhesion of many transformed cell lines and their growth in animals (Higgins et al. 1993; Narayanan et al. 1993). Finally, the putative oncogene *lyt-10*, which was generated by a chromosomal translocation in human B cell lymphomas, was identified as a NFκB1-related gene NFκB2 (Neri et al. 1991; Schmid et al. 1991; Bours et al. 1992). These observations provide evidence for the importance of NF-κB in cellular transformation and tumor growth.

The role of NF-κB in embryonic development is less understood. The NFκB1 (p105/p50) knockout mouse shows no defect in mouse development (Sha et al. 1995). However, it harbors defects in immunoglobulin class switching and defense against specific pathogens. In contrast, the *relA* (p65) knockout mouse is defective in embryonic development, specifically degeneration of liver cells, because of increased apoptosis (Beg et al. 1995a). RelB, another member of this family, shows no defect in embryo development when homozygously inactivated (Burkly et al. 1995; Weih et al. 1995); however, these mice show a loss of thymic dendritic cells, demonstrating a role for RelB in thymus development. In addition, these mice display other defects such as a lack of erythropoiesis in bone marrow, myeloid hyperplasia in bone marrow, and lung and liver inflammation (Weih et al. 1995).

Recently, mice without the c-*rel* gene have been generated, and these mice lack any defects in embryonic development (Kontgen et al. 1995). The mice display defects in proliferation of B and T cells in response to antigens, immunity, and IL-2 synthesis. Thus, to date the functions of the Rel/NF-κB family of proteins include functional differentiation of immune cells and organs, embryonic development specifically of the liver, cellular transformation and apoptosis. Additionally, it is clear that the members of the Rel/NF-κB family do not functionally compensate for one another, as mice lacking each gene are showing distinct defects. The definitive identification of the normal functions of each Rel/NF-κB member and specific Rel/NF-κB dimers will be greatly facilitated by individual and combination gene knockouts.

IκB family

Original identification and partial purification of IκB activity demonstrated that there are at least two distinct IκB proteins (IκBα and IκBβ) present in various cell extracts (Baeuerle et al. 1988; Baeuerle and Baltimore 1989; Ghosh and Baltimore 1990). Both of these proteins inhibited the DNA-binding activity of NF-κB (p50/p65) but not that of the p50 homodimer. Because many different

dimers of Rel/NF-κB proteins may coexist in a given cell type, their activity must be regulated independently or coordinately. This can be partially accomplished by the presence of different forms of IκB that differentially inhibit Rel/NF-κB dimers. There are at least seven mammalian IκB molecules identified with distinct and overlapping inhibitory specificity (Miyamoto and Verma 1995). These include IκBα, IκBβ, IκBγ, Bcl-3, p105, p100, and IκBR (Fig. 3) (Bours et al. 1990; Ghosh et al. 1990; Kieran et al. 1990; Ohno et al. 1990; Davis et al. 1991; Haskill et al. 1991; Meyer et al. 1991; Schmid et al. 1991; Inoue et al. 1992; Mercurio et al. 1992; Rice et al. 1992; Hatada et al. 1993; Henkel et al. 1993; Mercurio et al. 1993; Ray et al. 1995; Thompson et al. 1995). For example, IκBα inhibits complexes containing p65 and c-Rel, but IκBγ and Bcl-3 inhibit homodimers of p50 and p52 (for review, see Miyamoto and Verma 1995). In vivo, p105 and p100 are complexed with p50, p65, or c-Rel and retain them in the cytoplasm (Rice et al. 1992; Mercurio et al. 1993; Rice and Ernst 1993; Miyamoto et al. 1994a). Clearly, identification of the distribution of IκB members in association with different Rel/NF-κB proteins is critical in understanding their role in controlling the activity of various Rel/NF-κB dimers.

The IκB family of proteins, including *Drosophila* IκB Cactus, share conserved motifs referred to as ankyrin repeats, which are required for association with Rel/NF-κB proteins (Inoue et al. 1992; Hatada et al. 1993; for review, see Miyamoto and Verma 1995). An ankyrin repeat contains 30–33 amino-acids; the number of repeats varies from three to seven in the IκB family (Fig. 3). Not all ankyrin repeats are necessary for IκB function because ankyrin three of IκBα (pp40) can be mutated without any biochemical effect (Inoue et al. 1992). One important feature is that the ankyrin repeats of the IκB family are distinct from those of other ankyrin repeat-containing proteins, such as Ankyrin, CDC10, SWI6, and GABP-β (Nolan and Baltimore 1992). Therefore, not all ankyrin repeat-containing proteins can function as IκBs and some as yet undefined features of IκB ankyrin domain are essential for an efficient interaction with the NLS of Rel/NF-κB proteins. Conversely, not all NLSs are binding sites of IκB ankyrin motifs. The specific features of the NLS of Rel/NF-κB proteins that make it a substrate for IκB association are yet to be defined. Additionally, what makes different IκBs specific for various Rel/NF-κB dimers? For example, how does IκBα inhibit only dimers containing p65 or c-Rel, whereas Bcl-3 only inhibits homodimers of p50 or p52? The number of ankyrin repeats is not solely responsible for this specificity, because an increase in the number of repeats of IκBα does not alter specificity (Leveillard and Verma 1993). The specificity comes from the differences in affinity of interaction between IκBs and different Rel/NF-κB dimers. Consequently, when present in large excess, IκBα can inhibit p50 homodimers and an excess of IκBγ or Bcl-3 can inhibit NF-κB (Franzoso et al. 1992; Inoue et al. 1992; Zabel et al. 1993). It is also possible that in vivo post-translational modification of IκBs may change their affinity and, consequently, their specificity. It may be possible to define a "specificity domain" by swapping individual ankyrin repeats among the IκB family members and creating chimeric IκB proteins.

In addition to characteristic ankyrin repeats, the IκB family of proteins contains a carboxy-terminal acidic region (Naumann et al. 1993). When this region is removed from IκBα, the resulting mutant protein binds NF-κB in vitro but it cannot inhibit its DNA-binding activity (Inoue et al. 1992; Leveillard and Verma 1993; Naumann et al. 1993). Similarly, removal of the acidic region in the

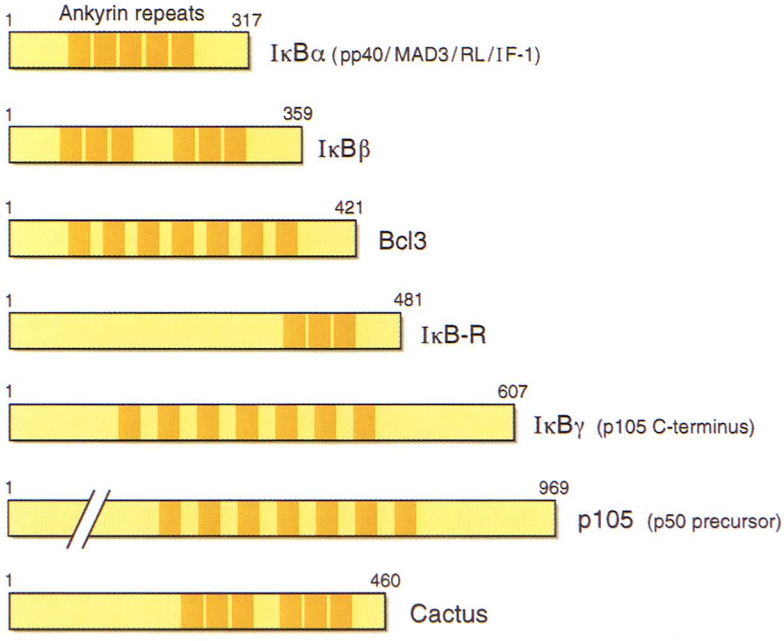

Figure 3. The IκB Family. The distinguishing feature of the IκB proteins is the presence of ankyrin repeats (orange boxes) whose numbers vary among the various members (ranging from 3 to 7). The ankyrin repeat motifs are required for protein–protein association. IκBγ is generated by an alternative mRNA from the gene encoding p105. Cactus is the functional homolog of IκB protein in *Drosophila*.

Bcl-3 protein disrupts its ability to inhibit the DNA-binding activity of the p50 homodimer (Leveillard and Verma 1993). Clearly, the ankyrin repeat region is necessary for association with NF-κB but is not sufficient for inhibition of the DNA-binding activity of NF-κB. However, it is not clear how the acidic domain of IκB is involved in inhibition of NF-κB DNA-binding activity. Additionally, recent studies show that the acidic region is important for proper interaction with NF-κB in vivo (Ernst et al. 1995; D. Van Antwerp, unpubl.). The acidic domain of IκBα may form an intermolecular interaction with the DNA-binding region of NF-κB (Ernst et al. 1995). X-ray crystallographic analyses of NF-κB/IκBα cocrystals will help define precise interactions between these two families of proteins.

Another feature of IκB is the presence in the carboxy-terminal domain of a Pro, Glu/Asp, Ser, and Thr-rich PEST sequence (Davis et al. 1991). Similar PEST sequences have been implicated in regulating protein half-life (Rechsteiner 1990). Deletion of the PEST sequence partially protects IκBα from degradation that is induced during NF-κB activation (Brown et al. 1995; Rodriguez et al. 1995). Because IκBβ is also degraded during NF-κB activation, it is likely that its PEST sequence plays a similar role in regulating its half-life (Thompson et al. 1995). However, the PEST sequence of Cactus is not required for its induced degradation in the *Drosophila* embryo (Belvin et al. 1995). Thus, the precise role of IκB PEST sequences in controlling the stability of IκB proteins merits further investigation.

Multiple signals activate NF-κB

One of the most intriguing aspects of the NF-κB/IκB system is the vast number and type of agents that can induce NF-κB activity in a variety of cell types (for review, see Grille et al. 1993; Baeuerle and Henkel 1994; Siebenlist et al. 1994; Miyamoto and Verma 1995). They include cytokines (TNFα, IL-1, and IL-2), bacterial LPS, virus infection (HIV-I, HTLV-I, and hepatitis B virus), viral proteins (tax, X, and E1A), stimulation of the antigen receptors of T and B cells, calcium ionophores, protein synthesis inhibitors, UV and x-ray irradiation, phorbol esters, and nitric oxide, among others. There is no obvious common secondary messenger that can be identified by inspecting the list of inducers. This may imply that NF-κB/IκB directly responds to many independent signaling molecules. However, most, if not all, of the NF-κB activating signals can be inhibited by antioxidants (Baeuerle and Henkel 1994). Hydrogen peroxide, a major reactive oxygen intermediate produced by the cell, activates NF-κB (Schreck et al. 1991). Therefore, the induction of reactive oxygen intermediates may have a central role in activation of NF-κB. It is of great interest and importance to identify how H_2O_2 induces NF-κB activation, especially the molecular targets of H_2O_2 involved in NF-κB activation.

Another unique feature of the regulation of NF-κB activity is the direct transcriptional activation of the inhibitor gene IκBα by NF-κB itself (Nolan et al. 1993; Sun et al. 1993; Chiao et al. 1994). The newly synthesized IκBα following the activation of NF-κB will immediately bind up free cytoplasmic NF-κB and inactivate its potential nuclear translocation. Furthermore, excess IκBα migrates into the nucleus by an unknown mechanism to remove active NF-κB from DNA and terminate its activity (Arenzana-Seisdedos et al. 1995). This nuclear NF-κB/IκBα complex may be transported back to the cytoplasm or degraded in the nucleus. Thus, NF-κB promotes continuous negative control of its own activity. Circumvention of this continuous negative regulation is possible only when continuous stimulation is provided resulting in sustained NF-κB activation. How other NF-κB-inducible IκB members, such as IκBβ, IκBγ, p105, p100, and Bcl-3, are involved in the negative NF-κB regulation is unclear. Together with IκBα they may play an important role in rapid inactivation of the transcription of the target genes when the activating signals are withdrawn. Furthermore, such a system will allow immediate reactivation of the NF-κB because NF-κB/IκB complexes are continuously regenerated in the cytoplasm. Recently, two research groups have shown that immunosuppression by glucocorticoids may be the direct outcome of the increased transcriptional activation of the IκBα gene (Auphan et al. 1995; Scheinmann et al. 1995). It appears that the newly synthesized IκBα protein rapidly reassociates with the newly released NF-κB, thereby markedly reducing the amount of NF-κB translocated to the nucleus for the activation of cytokine genes. Additionally, immunosuppression and down-regulation of cytokine gene expression may require the newly synthesized IκBα to translocate to the nucleus and bind to the p65 subunit of the NF-κB complex associated with DNA, thereby effectively preventing gene activation. An immediate prediction from these results will be that cells lacking the IκBα gene (IκBα −/−, see below) will not inhibit NF-κB activity in response to glucocorticoids. Since retinoids also inhibit NF-κB activation, it will be interesting to know if the mechanism of inhibition also involves transcriptional activation of IκBα.

Mice, where the IκBα gene has been deleted by homologous recombination, have been generated (Beg et al. 1995b). Although normal at birth, IκBα −/− mice exhibit severe runting, skin defects, and extensive postnatal granulopoiesis and typically die by 8 days. Analysis of the hematopoietic tissues from these mice show elevated levels of both nuclear NF-κB and mRNAs of several genes regulated by NF-κB. These phenotypic abnormalities likely arise from loss of IκBα protein, because mice lacking both IκBα and the p50 subunit of NF-κB show dramatically delayed onset of abnormalities. In contrast to hematopoietic cells, IκBα −/− embryonic fibroblasts show minimal constitutive NF-κB, but normal signal-dependent NF-κB activities that are concomitant with IκBβ degradation. Furthermore, Beg et al. (1995b) demonstrate that IκBα is, however, required for postinduction repression of NF-κB in fibroblasts. These results also clearly demarcate the distinct roles of IκBα and IκBβ proteins.

IκB is the target of incoming signals

There are many intermediate signaling molecules besides H_2O_2 implicated in the activation of NF-κB in different systems. These include tyrosine phosphorylation events, the Ras–Raf pathway, PK-C, and double-stranded RNA (dsRNA)-dependent kinase (for review, see Miyamoto and Verma 1995). Although phosphorylation of p65 by an associated serine kinase has been implicated in the activation of NF-κB (Hayashi et al. 1993), in most cases the ultimate target of these signaling molecules is IκB (for review, see Beg and Baldwin 1993; Baeuerle and Henkel 1994; Israel 1995; Collins et al. 1995; Miyamoto and Verma 1995). This conclusion is based on early observations suggesting that modification must occur on IκB rather than NF-κB (Baeuerle et al. 1988). First, cytoplasmic NF-κB bound to IκB has intrinsic DNA-binding activity, because the release of IκB by detergents allows NF-κB DNA-binding. Second, the DNA-binding activity of NF-κB obtained by detergent treatment of the cytoplasmic NF-κB/IκB complex is comparable to that appearing in the nucleus following stimulation. Third, the DNA-binding activity of nuclear NF-κB can be inhibited by exogenously added IκB. Finally, IκB activity is not recovered from the cytoplasm following stimulation. Additionally, in vitro studies using partially purified IκB showed that direct phosphorylation of IκB by various kinases, including PK-C, PK-A, and heme-regulated eIF-2, resulted in inactivation of IκB activity (Shirakawa and Mizel 1989; Ghosh and Baltimore 1990). When the substrate for these kinases was the NF-κB/IκB complex, phosphorylation of IκB resulted in the release of NF-κB and the appearance of the NF-κB DNA-binding activity. From these observations, a model was proposed in which phosphorylation of IκB results in its dissociation from NF-κB, thereby freeing NF-κB to translocate to the nucleus and bind κB sites (Baeuerle 1991). Although recent experiments demonstrate that this model needs further embellishments to explain in vivo activation of NF-κB, it is certain that IκB phosphorylation is an integral and obligatory step in NF-κB activation in vivo.

Induced phosphorylation of IκB

Because many kinases can phosphorylate and inactivate IκB in vitro as described above, many different kinases possibly inactivate IκB by phosphorylating various sites in vivo. Alternatively, a specific IκB kinase may be responsible for IκB phosphorylation and inactivation in vivo. Finally, it is possible that phosphorylation of IκB is not a regulatory step in vivo following stimulation. However, through the use of antibodies and phosphatase inhibitors, phosphorylation of IκBα is observed in many cell types following stimulation (Beg et al. 1993; Cordle et al. 1993; Mellits et al. 1993). Importantly, this phosphorylation event precedes NF-κB activation. Induced phosphorylation of IκBα reduces its mobility in polyacrylamide gel electrophoresis and, consequently, slower migrating IκBα species can be detected by Western blot analysis of cytoplasmic extracts. The retardation of the migration of IκBα is attributable to phosphorylation because phosphatase treatment results in its faster migration. More than one phosphorylated species can often be detected, indicating that more than a single IκBα phosphorylation event is induced. How many phosphorylation events are induced? Whether the phosphorylation events are different for various activators, and whether IκB phosphorylation is essential for NF-κB activation remain unanswered questions (see below).

The induced IκBα phosphorylation sites have not been mapped by conventional two-dimensional peptide mapping, because of the very rapid degradation of the phosphorylated IκBα proteins (Beg et al. 1993; Brown et al. 1993; Cordle et al. 1993; Henkel et al. 1993; Mellits et al. 1993; see below). Fortunately, an independent approach, namely mutagenesis of IκBα, resulted in the identification of an amino-terminal deletion mutant that did not undergo phosphorylation when stably expressed in a reporter cell (Brockman et al. 1995; Brown et al. 1995). This observation led to the discovery of the potential key phosphoacceptor residues in the amino-terminal domain. When Ser-32 and Ser-36 in this amino terminus were mutated to alanine individually (Brown et al. 1995) or in combination (S32/36A), IκBα failed to undergo phosphorylation following stimulation (Brown et al. 1995; Traenckner et al. 1995; Whiteside et al. 1995; S. Miyamoto, D. Van Antwerp, and J. Stevenson, unpubl.). This does not constitute proof, but it does provide strong evidence that these serines are phosphorylated during NF-κB activation. Accordingly, limited protease digestion of the NF-κB/IκBα complex demonstrates that the amino terminus of IκBα is accessible to proteases (Arenzana-Seisdedos et al. 1995) and therefore to other proteins, including, perhaps, IκBα kinases. Because the S32/36A IκBα mutant is resistant to phosphorylation induced by various stimuli, including okadaic acid, TNF, and phorbol ester (TPA), different signaling pathways seem to result in the phosphorylation of these same sites (Traenckner et al. 1994; Brockman et al. 1995). Furthermore, the results imply that the kinase or kinases that phosphorylate IκBα upon stimulation with a variety of inducers recognize the same Ser residues. Therefore, the IκBα kinase may be a unique or novel class of related kinases rather than many different types of kinases with distinct recognition sites, as was implied by the in vitro phosphorylation experiments mentioned above. A further indication that in vitro phosphorylation sites are unrelated to events in vivo is that the in vitro kinase reaction results in dissociation of IκB from NF-κB whereas in vivo phosphorylation does not (see below). Thus, Ser-32 and Ser-36 are most likely the induced phosphoacceptor sites in vivo, and further experimentation is required to define whether phosphorylation occurs on both sites coordinately or independently. Incidentally, in IκBβ, there are equivalent serine residues, Ser-19 and Ser-23, which could serve as the sites of induced phosphorylation.

Is there a phosphatase specific for removing the induced phosphate groups from IκBα? It is well known that phosphorylated IκBα can be detected more easily if cell

extracts are treated with various phosphatase inhibitors. If unstimulated cells are treated with okadaic acid, an inhibitor of the phosphatase PP2A, some phosphorylated IκBα is observed (Sun et al. 1994; Traenckner et al. 1994). This result indicates either that IκBα in its unstimulated state is undergoing continuous phosphorylation and dephosphorylation events or that the inhibition of dephosphorylation by okadaic acid of some other upstream signaling molecule results in activation of IκBα kinases. The former possibility indicates the presence of a specific phosphatase that is actively removing phosphate groups from IκBα. Such a phosphatase would negatively regulate NF-κB activity, and in some cases, inhibition of this phosphatase might be induced during NF-κB activation. Although the presence of IκBα phosphatase is hypothetical at present, its identification may provide an important target for drug development to control NF-κB/IκBα system.

Basal phosphorylation of IκBα

Although not stressed in the literature, IκBα is a phosphoprotein in unstimulated cells (Davis et al. 1991; Kerr et al. 1991; Barroga et al. 1995; Didonato et al. 1995). The nature of the phosphorylation sites, the kinases that phosphorylate it, and the functional significance of this phosphorylation event have been elusive for many years. Our recent studies demonstrate that the basal phosphorylation of IκBα occurs at the carboxy-terminal casein kinase II (CKII) sites in the PEST region and is mediated by CKII (Barroga et al. 1995). Although there are five potential Ser/Thr CKII phosphorylation sites, the preferred site appears to be Ser-293. An IκBα mutant (IκBα–MutF), where all five sites are mutated to alanine, cannot be phosphorylated in vitro either by cellular extracts or by purified CKII. More importantly, IκBα–MutF is not phosphorylated in vivo when transiently expressed in 293 cells, a human embryonic kidney cell line. IκBα–MutF forms a complex with p65, indicating that the basal phosphorylation of IκBα is not required for NF-κB association. It is also clear that basal phosphorylation is not required for induced IκBα phosphorylation and degradation, because IκBα–MutF expressed stably in HeLa cells undergoes phosphorylation and degradation upon TNFα stimulation. Interestingly, free IκBα–MutF has a half-life twice as long as the wild type when transiently overexpressed in 293 cells. Consequently, IκBα–MutF can act as a transdominant negative mutant in that it inhibits signal-induced activation of endogenous NF-κB because of overabundance of free inhibitor proteins. In addition, excess wild-type IκBα can also hinder the activity of newly activated NF-κB proteins. The presence of free IκBα in unstimulated cells would prevent rapid inducibility and reduce the sensitivity of the NF-κB system. Consequently, there must be a mechanism for removing any unbound wild-type IκBα efficiently from the cell. Such a mechanism could require basal phosphorylation because it has been demonstrated that free wild-type IκBα is basally phosphorylated and short lived (Scott et al. 1993; Sun et al. 1993), whereas free IκBα–MutF is unphosphorylated and has a longer half-life (J.K. Stevenson, unpubl.). In the *Drosophila* embryo, free Cactus also has a short half-life, and its degradation requires the carboxy-terminal PEST sequence where the basal phosphorylation sites reside (Belvin et al. 1995). Deletion of the PEST sequence from IκBα also results in a longer half-life of the free mutant protein. Taken together, a plausible function of the basal phosphorylation in the PEST region of IκBα is to target excess free IκBα efficiently to a degradation machinery and ensure the rapid inducibility and preserve the sensitivity of the NF-κB system.

Induced degradation of IκBα

As mentioned above, IκBα undergoes complete degradation following stimulation but prior to NF-κB activation (Beg et al. 1993; Brown et al. 1993; Cordle et al. 1993; Henkel et al. 1993; Mellits et al. 1993; Chiao et al. 1994). This induced degradation is rapid and in some cell types it is complete within ~10 min. It is also extensive and produces no obvious intermediates. Thus, the induced IκBα degradation is a remarkably efficient process that can be inhibited if cells are treated with cell-permeable serine protease inhibitors, such as TPCK (Henkel et al. 1993; Chiao et al. 1994). More importantly, NF-κB activation is also inhibited (Henkel et al. 1993). This observation suggested that the degradation of IκBα is an obligatory step in the activation of NF-κB. However, in this study, it was not certain whether these protease inhibitors only inhibited IκBα degradation or also IκBα phosphorylation. Additional experiments demonstrated that pretreatment of cells with TPCK or TLCK does inhibit IκBα phosphorylation in vivo (Mellits et al. 1993; Finco et al. 1994; Miyamoto et al. 1994b; Sun et al. 1994; Alkalay et al. 1995; Didonato et al. 1995). Use of other protease inhibitors, namely, cell-permeable peptide aldehydes such as calpain inhibitor I, clearly demonstrated that IκBα phosphorylation can be induced in the absence of its degradation (see below).

Thus, these observations raise a number of mechanistic questions about NF-κB activation: (1) What is the nature of the kinase (IκBα kinase) that phosphorylates IκBα upon induction? (2) Does IκBα phosphorylation result in its dissociation from NF-κB? (3) What is the signal for IκBα degradation? (4) What is the substrate for IκBα protease? (5) What is the IκBα protease? (6) And what is the sequence of events leading to NF-κB activation? If phosphorylation of IκBα causes dissociation of NF-κB, as the original model suggested (Baeuerle 1991), protease inhibitors would have no effect on NF-κB activation. However, peptide aldehyde inhibitors block NF-κB activation and coimmunoprecipitation studies demonstrate that when degradation is blocked the phosphorylated form of IκBα (IκBα-P) is still complexed with NF-κB in the cytoplasm (Miyamoto et al. 1994b; Palombella et al. 1994; Traenckner et al. 1994; Brockman et al. 1995; Brown et al. 1995; Didonato et al. 1995; Lin et al. 1995). Thus, the induced degradation, not the phosphorylation event, results in liberation of free NF-κB. The lack of induced phosphorylation and degradation of the S32/

36A mutant of IκBα mentioned above provides evidence that induced IκBα phosphorylation is required and provides a signal for degradation. The phosphorylation event is required for an additional modification of IκBα, namely, multiubiquitination (Chen et al. 1995; M. Roff, J. Thompson, M.S. Rodriguez, J.-M. Jacque, F. Baleux, F. Arenzana-Seisdedos, and R.T. Hay, in prep.). The ubiquitinated IκBα remains associated with NF-κB and is specifically degraded by 26S proteasome. Ubiquitin is conjugated at Lys-21 and Lys-22 in the amino-terminal region of IκBα (Rodriguez et al. 1995; Sherer et al. 1995). Thus, the sequence of events leading to NF-κB activation may require phosphorylation of IκBα at Ser-32 and Ser-36 residues, followed by phosphorylation-dependent multiubiquitination of IκBα at Lys-21 and Lys-22 and degradation of IκBα by ubiquitin-dependent proteasome and finally the release of free NF-κB transcription factor (Fig. 4).

Basal degradation of IκBα

It is important to note that IκBα in its unstimulated state is continuously turning over (Henkel et al. 1993; Rice and Ernst 1993; Miyamoto et al. 1994a). The half-life of IκBα is ~2.5 hr in the 70Z/3 murine pre-B cell line, making it a very unstable protein (Miyamoto et al. 1994a). On the other hand, the half-life of Rel/NF-κB is much longer in the same cells (Miyamoto et al. 1994a). This is in agreement with an earlier observation that NF-κB is regulated by a labile inhibitor (Sen and Baltimore 1986). Additionally, it explains why inhibition of protein synthesis results in NF-κB activation (Sen and Baltimore 1986). If IκBα is turning over faster than NF-κB, the lack of IκBα synthesis will eventually lead to the presence of free NF-κB.

These observations raise some important questions: Is the basal IκBα protease the same as or different from the

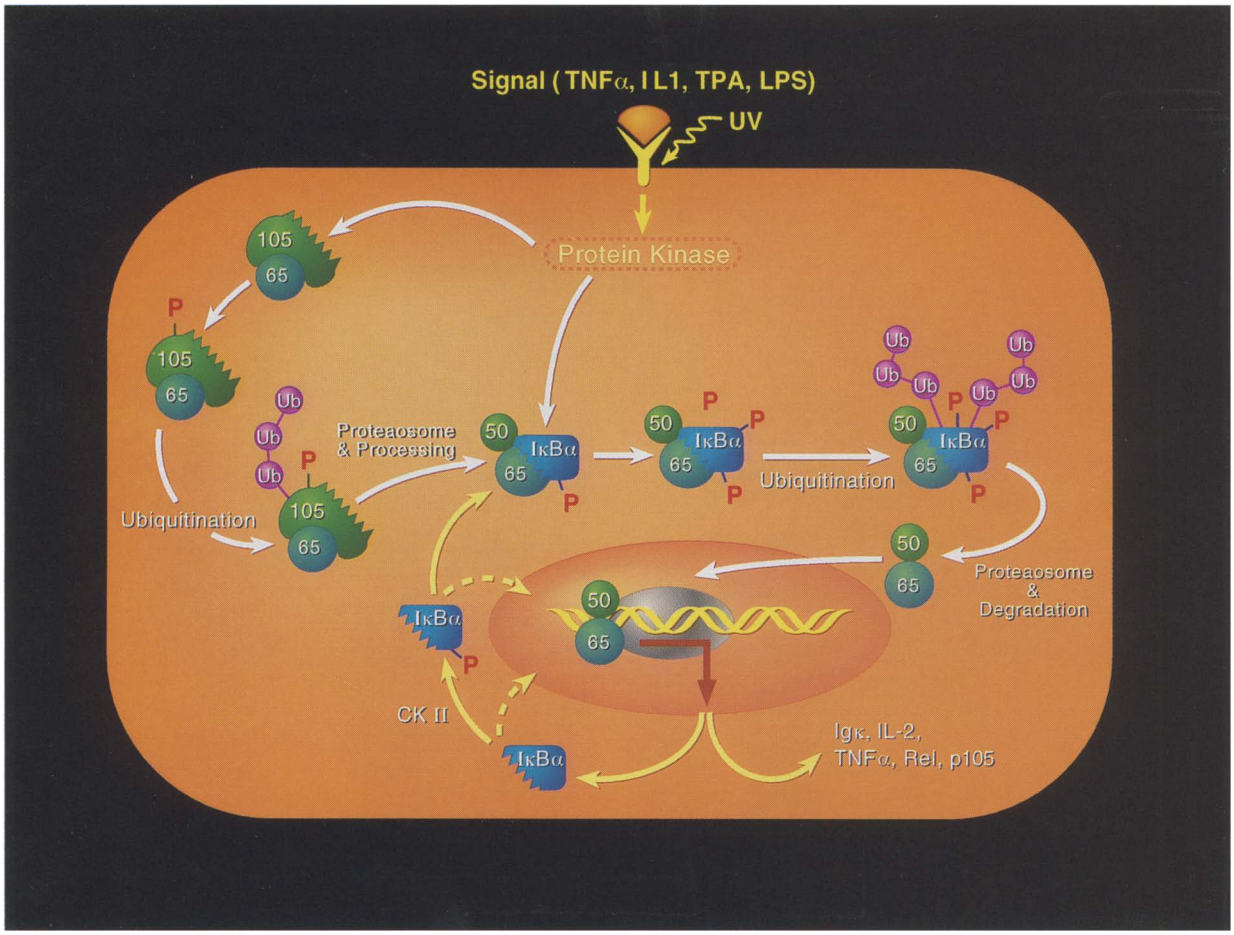

Figure 4. Model for NF-κB regulation. In response to external signals a protein kinase is induced that can phosphorylate both p105 present in association with p65 and IκBα present in association with p50/p65. The phosphorylated p105/p65 and p50/p65/IκBα are then substrates for ubiquitination followed by processing of p105 to p50 and degradation of IκBα by proteasomes. The p50/p65 complex then translocates to the nucleus activating genes containing the κB site, including IκBα. The IκBα protein is modified by phosphorylation with CKII and can form a complex with processed p50/p65 complex and be retained in the cytoplasm until a new signal is provided. The newly synthesized IκBα protein can also enter the nucleus. There is no formal proof to rule out if the newly processed p50/p65 complex does not go directly to the nucleus without association with IκB, but the kinetics of processing are slower than NF-κB activation.

induced protease? That is, does the basal IκBα protease require phosphorylation at Ser-32 and Ser-36 as does the induced protease? And if induced phosphorylation is not necessary, is it possible to activate NF-κB by modulating the activity of the basal protease without affecting the IκBα kinase? These questions may be best tested by using WEHI–231 murine B cells, where NF-κB is constitutively active (Sen and Baltimore 1986).

The mechanism of constitutive NF-κB activation in this cell type has remained unknown for many years. We and others have shown that the constitutively active Rel/NF-κB complex in WEHI–231 cells is a p50/c-Rel dimer (Rice and Ernst 1993; Grumont and Gerondakis 1994b; Liou et al. 1994; Miyamoto et al. 1994c). The constitutive activity of the p50/c-Rel dimer is not attributable to H_2O_2 production because treatment of WEHI–231 cells with an antioxidant PDTC has no effect, whereas the antioxidant does inhibit LPS-induced NF-κB activation in 70Z/3 cells (S. Miyamoto, unpubl.). The DNA-binding activity of the p50/c-Rel dimer is inhibited by exogenously added IκBs, showing that p50/c-Rel retains sensitivity to IκB proteins (Miyamoto et al. 1994a). Newly synthesized c-Rel in WEHI–231 cells is complexed with IκB proteins, such as IκBα and p105 (Rice and Ernst 1993; Miyamoto et al. 1994a). Furthermore, IκB proteins associated with c-Rel are cytoplasmic, demonstrating that IκBs inhibit c-Rel nuclear translocation (Miyamoto et al. 1994a). Surprisingly, we found that only ~10%–20% of c-Rel is free of IκB proteins and detectable in the nucleus (Miyamoto et al. 1994a). In addition, cytoplasmic IκBα is constitutively turning over with a half-life of ~30 min in WEHI–231 cells (Miyamoto et al. 1994a). Slowing this half-life by protease inhibitors, including calpain inhibitors, leads to the loss of p50/c-Rel dimers from the nucleus (Miyamoto et al. 1994a; S. Miyamoto, unpubl.). The induced degradation machinery is intact in these cells, because stimulation of these cells with LPS or TPA results in enhanced IκBα degradation.

From these observations we propose that enhanced basal IκBα degradation contributes to constitutive p50/c-Rel activation in WEHI–231 cells. Taken together, enhancement of basal IκBα degradation in the absence of external stimuli can result in constitutive NF-κB activation. It is of interest to determine whether the amino-terminal deletion S32/36A and CKII site mutants of IκBα can inhibit constitutive p50/c-Rel activity in WEHI–231 cells. Alternatively, these mutant proteins may be degraded as fast as endogenous IκBα in WEHI–231 cells. Such studies could help to determine whether induced and basal phosphorylation of IκBα are essential for constitutive NF-κB activation in WEHI–231 cells.

Nuclear translocation of NF-κB

An aspect of the NF-κB system that has not been studied extensively is the kinetics of nuclear translocation of NF-κB proteins following activation. Although complete IκBα degradation and maximum DNA-binding activity appears in <10 min following stimulation in some cells, the amount of NF-κB proteins that translocate into the nucleus within the same period is <10%–20% of total NF-κB proteins (Miyamoto et al. 1994a). What happens to the majority of NF-κB that has been freed from IκBα? Some NF-κB proteins may be associated with other IκB proteins, such as IκBβ, Bcl-3, p105, and p100. Also, the nuclear translocation machinery may reach a saturation point. Additionally, there may be other unexplored regulatory steps important for their nuclear translocation. Nuclear translocation of the *Drosophila* NF-κB member Dorsal requires phosphorylation at the conserved PK-A site (Norris and Manley 1992). Furthermore, the oncoprotein v-Rel is constitutively nuclear if the PK-A site is mutated from a serine to an alanine (Mosialos et al. 1991). Thus, a phosphorylation or dephosphorylation event at the conserved PK-A site may provide a regulatory event for NF-κB nuclear translocation following dissociation from IκBα. The NF-κB subunit p65 (RelA) and c-Rel have been shown to be phosphorylated following activation (Li et al. 1994; Naumann and Scheidereit 1994), but the phosphorylation sites are not defined in these studies. Thus, the mechanism of NF-κB nuclear translocation following IκBα degradation may involve a phosphorylation or dephosphorylation event and provide a critical step in NF-κB regulation.

Once in the nucleus, the NF-κB proteins not only bind to the decameric DNA sequence but also interact with many other proteins. For example, c-Rel has been shown to bind directly to TATA binding protein (TBP) and TFIIB (Kerr et al. 1993; Xu et al. 1993). Direct association between c-Rel and TBP is essential for transcriptional activation. Using a yeast two-hybrid system, we have recently found that c-Rel also interacts with one of the RNA polymerase II subunits (K. Tashiro and I.M. Verma, unpubl.). NF-κB (p50/p65) alone is not sufficient to activate the transcription of human interferon-β (IFN-β) gene, even though it binds to the specific κB site. It requires a high-mobility-group protein [HMG-I(Y)] as a coactivator for transcriptional activation (Thanos and Maniatis 1992). The Rel/NF-κB/Dorsal proteins can also function as repressors, either alone like the p50 homodimer (Schmitz and Baeuerle 1991; Franzoso et al. 1992; Kang et al. 1992) or in association with another protein like the *Drosophila* switch protein (DSP-1) (Lehming et al. 1994). Additionally, there are other activator proteins like the helix–loop–helix (HLH) family member, C/EBP, or steroid receptors, which can interact with the NF-κB proteins to activate, repress, enhance, or decrease transcription of various genes (Miyamoto and Verma 1995). Thus, NF-κB proteins, once entered in the nucleus, are subjected to additional levels of control.

Conclusions and perspectives

The distribution of the Rel/NF-κB/IκB system is widespread and is receptive to many independent extracellular and intracellular signals. Consequently, the types of genes that are regulated by this family of transcription factors are diverse, demonstrating their significance in a wide variety of cellular functions. A role of NF-κB in cell

proliferation is suggested by activation of p53, c-*myc*, and c-H-*ras* genes which are important for cell cycle progression. NF-κB may also play a role in apoptosis because p65 and c-Rel have been implicated in apoptosis of embryonic liver cells and chicken bone marrow cells, respectively (Beg et al. 1995a). However, the role of NF-κB in apoptosis is not universal. A recent study shows that NF-κB is not involved in TNFα-induced apoptosis of human 293 cells (Hsu et al. 1995). What is evident are the fundamental roles of Rel/NF-κB family proteins in immune responses, thymus development, inflammatory and acute phase responses, and embryonic development. Definitive answers to the biological roles of this family of transcription factors will likely come from homozygous knockout studies of Rel/NF-κB genes individually and in combination.

Much of the effort in the field has been devoted to the dissection of the molecular events associated with the release of NF-κB from the cytoplasmic NF-κB/IκBα complex. Consequently, a large body of information describes clear and sequential post-translational modification events: phosphorylation of IκBα probably at Ser-32 and Ser-36, followed by multiubiquitination at residues Lys-21 and Lys-22, degradation of IκBα by 26S proteasome, and liberation of free NF-κB to translocate into the nucleus. To determine how phosphorylated IκBα is selectively targeted for proteolysis without concomitant destruction of the associated NF-κB requires further investigation. IκBα is also continually degraded in the unstimulated cells and the identification of the protease responsible for basal IκBα degradation needs exploration. Future studies will fill in additional events that are missing in this simplistic model, such as involvement of phosphatases, mechanism of ubiquitination of IκBα, and regulation of nuclear translocation of free NF-κB.

One of the most pursued proteins for study in the NF-κB system, the IκBα kinase, remains elusive. This kinase must respond to a wide variety of signals and should be situated downstream of and possibly directly regulated by H_2O_2. Besides IκBα, there are many other members of the IκB family of proteins. How their activities are regulated during NF-κB activation is not clearly understood. Some signals that degrade IκBα and IκBβ also induce processing of p105 and/or p100 to p50 and p52, respectively. Additionally, not all signals that cause IκBα phosphorylation and degradation induce IκBβ degradation. Finally, the regulation of Bcl-3 activity in vivo is almost entirely unknown. Significant amounts of Bcl-3 and p50 homodimer can be found in the nucleus of cells, such as murine B cells (Liou et al. 1994; Miyamoto et al. 1994c). It is not known whether a p50 homodimer and Bcl-3 are present in a complex in the nucleus of these cells. However, Bcl-3 regulation of the cytoplasm/nuclear localization of p50 and p52 seems very distinct from that of other IκB proteins (Siebenlist et al. 1994). This difference of regulation may be related to the unique coactivator activity of Bcl-3 in addition to the IκB activity.

The Rel/NF-κB family of transcription factors are important regulators of viral transcription, including that of the human immunodeficiency virus. NF-κB also plays an important role in transformation induced by another human pathogenic virus, the human T cell leukemia virus-I. In addition, some members of the NF-κB protein family, such as c-Rel, Lyt-10, and Bcl-3, are implicated in human B cell lymphomas. The involvement of Rel/NF-κB family in other types of human cancers is likely, especially because p65 is important for tumor cell adhesion and growth. In addition, it is well documented that these transcription factors play pivotal roles in immune, inflammatory, and acute phase responses. Failure or overreaction of these responses can result in serious conditions including toxic shock and death. Thus, drug development for specific attenuation of Rel/NF-κB activity will help to alleviate human disease states associated with this family of transcription factors, including AIDS and cancer. The targets of such drugs may include reactive oxygen intermediates, IκBα kinases, ubiquitination machinery, 26S proteasomes, basal IκBα proteases, specific nuclear translocation machinery, or Rel/NF-κB/IκB complexes themselves. With the rapid rate of progress in the field, development of such important drugs should be forthcoming in the near future.

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