Mechanistic Aspects of NF-κB Regulation: The Emerging Role of Phosphorylation and Proteolysis

Review

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Members of the NF-κB transcription factor family serve as a prototypical model of inducible transcription factors. As discussed below, NF-κB activity is primarily regulated by a group of structurally related proteins collectively referred to as IκB (for recent reviews on NF-κB and IκB, see Beg and Baldwin, 1993; Gilmore and Morin, 1993; Siebenlist et al., 1994; Baeuerle and Henkel, 1994; Thanos and Maniatis, 1995). The NF-κB transcription factor family is an important component in a variety of biological processes, most notably inflammation and immune responses. The requirement for NF-κB in these processes is indicated by its ability to regulate genes whose products are critical for these cellular events. These products include cytokines, immunoreceptors, cell adhesion molecules, and acute phase proteins (Siebenlist et al., 1994; Baeuerle and Henkel, 1994). In addition, recent research has shown dramatically that mice containing targeted disruptions of NF-κB subunits are compromised in various aspects of immune function and inflammatory responses (Weih et al., 1995; Sha et al., 1995; Burkly et al., 1995; Kontgen et al., 1995; also see Thanos and Maniatis, 1995). A number of pathogenic viruses, including the human immunodeficiency virus, also subvert NF-κB activity for the expression of essential viral genes (Nabel and Baltimore, 1987; Siebenlist et al., 1994; Baeuerle and Henkel, 1994). Finally, genetic alterations in the structure or expression of genes encoding NF-κB and IκB family members can render these proteins oncogenic, demonstrating an intimate relationship between NF-κB function and normal cellular proliferation (Siebenlist et al., 1994; Baeuerle and Henkel, 1994). In this review, we discuss the mechanisms regulating NF-κB activity. A particular emphasis has been placed on recent studies, which more clearly define how various modifications to IκB, specifically inducible phosphorylation, ubiquitination, and proteasome-driven degradation, contribute to NF-κB activation.

NF-κB Is an Inducible Transcription Factor

Research performed in the late 1980s elegantly demonstrated the inducible nature of NF-κB. These studies revealed that NF-κB is primarily regulated by its localization within the cell (Baeuerle and Baltimore, 1988a). In most circumstances, NF-κB is sequestered in the cytoplasm through its interaction with a group of inhibitory molecules termed IκB (Baeuerle and Baltimore, 1988b). Treatment of cells with various inducers, including tumor necrosis factor (TNF), interleukin-1 (IL-1), lipopolysaccharide (LPS), phorbol esters, and serum growth factors results in the dissociation of IκB from NF-κB (Siebenlist et al., 1994; Baeuerle and Henkel, 1994). IκB displacement exposes the nuclear localization signal of NF-κB, thereby allowing translocation of the transcription factor into the nucleus, where it modulates gene expression (Beg et al., 1992; Ganchi et al., 1992; Zabel et al., 1993). Thus, the dissociation of IκB from NF-κB is the critical limiting step in the activation process. The translocation of NF-κB into the nucleus is remarkably rapid, usually occurring within minutes of cellular stimulation. This rapidity, and the observation that activation does not require protein synthesis (Sen and Baltimore, 1986), strongly implied that signal transduction pathways are directly responsible for the removal of IκB from NF-κB. Another important property of NF-κB regulation is that activation is typically transient. Shortly after stimulation of cells with inducers of NF-κB, newly formed NF-κB–IκB complexes begin to reaccumulate in the cytoplasm (Beg et al., 1993; Brown et al., 1993; Cordle et al., 1993; Henkel et al., 1993; Sun et al., 1993). Concurrently, NF-κB binding activity within the nucleus subsides. In the years since the initial characterization of the inducible nature of NF-κB, intense research has centered on elucidating the signaling events responsible for the dissociation of IκB from NF-κB in vivo. Moreover, investigators have worked to comprehend the mechanisms governing the transient nature of activation. A crucial step toward understanding these complex events was the characterization of proteins that comprise both the NF-κB and the IκB families.

NF-κB and IκB Are Each Composed of a Family of Proteins

Classic NF-κB is a heterodimer of a 50 kDa subunit (NF-κB1 or p50) and a 65 kDa subunit (RelA or p65) (Baeuerle and Baltimore, 1989; Kawakami et al., 1988). Cloning of the cDNAs encoding these proteins revealed two important features (Ghosh et al., 1990; Kieran et al., 1990; Nolan et al., 1991; Ruben et al., 1991). First, both proteins are homologous to the product of the c-Rel proto-oncogene and second, the 50 kDa subunit is derived from the amino terminal portion of a precursor protein, p105 (see Figure 1). Unexpectedly, p105 was also found to contain a cluster of so-called ankyrin or cell cycle repeats at its carboxyl terminus. The homology that exists among p50, p65, and c-Rel is within an approximately 300 aa region, the Rel homology domain, which is common to all NF-κB family members (Figure 1). This essential region is multifunctional, controlling NF-κB dimerization, interaction with IκB, DNA binding, and it also contains the nuclear localization signal. The two other protein subunits within the NF-κB family are RelB and p52 (NF-κB2) (Schmid et al., 1991; Ryseck et al., 1992; Bours et al., 1992; Mercurio et al., 1992). Like p50, p52 is initially expressed as an ankyrin repeat–containing precursor protein termed p100. Proteins that constitute the NF-κB family form a variety of homo- and heterodimers, which have varying affinities for related NF-κB DNA binding sites. The structure of one of these NF-κB complexes bound to DNA, specifically the Rel homology domain of p50 homodimers, has recently

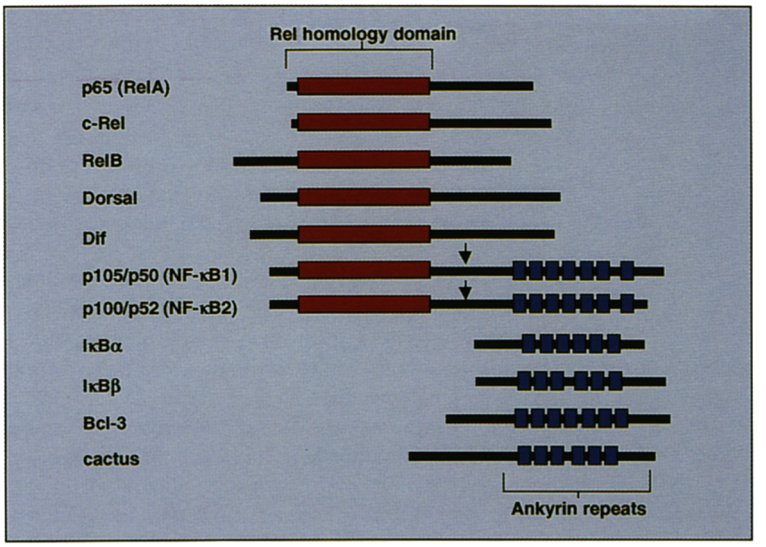

Figure 1. Schematic Diagram of Members of the NF-κB and of the IκB Family of Proteins

The Rel domain (shown in red) is approximately 300 aa in length, is found in all NF-κB family members, and is required for dimerization, DNA binding, interaction with IκB, and contains the nuclear localization signal. Ankyrin repeats (shown in blue) are roughly 33 aa in length, are found in all IκB family members, and are necessary for the interaction of IκB with NF-κB. Processing sites for p105/p50 and p100/p52 are indicated with arrows. All proteins are of mammalian origin except for dorsal, Dif, and cactus, which are found in Drosophila. Refer to the text for details.

been determined by X-ray crystallography techniques (Ghosh et al., 1995; Muller et al., 1995). The two complementary studies impressively reveal a DNA binding domain for NF-κB unrelated to those found in previously characterized proteins. During binding, p50 homodimers envelop the DNA through a series of interactions dispersed along the Rel homology domain, resulting in a three-dimensional structure that resembles a butterfly.

Most of the various homo- and heterodimeric NF-κB complexes detected in vivo are regulated by IκB-mediated cytoplasmic retention. The cloning of a major form of IκB, IκBα, revealed that it contained a number of ankyrin repeats similar to those found in the precursor p105 (see Figure 1) (Haskill et al., 1991). Ankyrin repeats, each of which are roughly 33 aa in length, are found in a variety of unrelated proteins and have been implicated in protein–protein interactions (Thompson et al., 1991). Consistent with this role, the ankyrin repeats of IκB are required for interaction with NF-κB (Inoue et al., 1992; Hatada et al., 1992, 1993; Bours et al., 1993). A number of papers have provided conclusive data that IκBα inhibits NF-κB activity by sequestering it in the cytoplasm (Beg et al., 1992; Ganchi et al., 1992; Zabel et al., 1993). Subsequent studies have demonstrated that p105 and p100 can similarly function as IκBs (Rice et al., 1992; Mercurio et al., 1992; Naumann et al., 1993; Scheinman et al., 1993). The processing of p105 and p100 then yields active p50- and p52-containing NF-κB complexes that have the capacity to translocate into the nucleus. Therefore, the precursors to p50 and p52 can be viewed as members of the IκB family, while the processed proteins belong to the NF-κB family. There are at least two other mammalian members of the IκB family, IκBβ and Bcl-3, both of which also contain ankyrin repeats. The cloning of IκBβ eluded researchers until very recently (Thompson et al., 1995). This study confirmed that IκBβ functions similarly to IκBα, p105, and p100 in its ability to regulate NF-κB by cytoplasmic retention. However, as discussed later, it was also demonstrated that IκBβ exhibits unique characteristics that functionally distinguish it from IκBα. In contrast with the other forms of IκB, Bcl-3 is predominantly nuclear and may actually enhance NF-κB activity by serving as a transcriptional coactivator for p50 or p52 homodimers (Fujita et al., 1993; Bours et al., 1993). Although studies have implicated each form of IκB discussed above as a potential target in the control of NF-κB function, the majority of the research has focused on the roles of IκBα, p105, and, to a lesser extent, IκBβ, in NF-κB regulation. For this reason, our discussion will primarily be limited to these molecules.

A system strikingly similar to NF-κB and IκB exists in Drosophila. In this invertebrate, two Rel-related DNA binding proteins, dorsal and Dif, are regulated by cytoplasmic/nuclear localization (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989; Ip et al., 1993). Cytoplasmic retention of dorsal (and most likely Dif) is modulated via interaction with an ankyrin repeat–containing inhibitor known as cactus (Geisler et al., 1992; Kidd, 1992). Dorsal plays a critical role in dorsoventral patterning during early Drosophila embryogenesis. In addition, dorsal and Dif are implicated as important mediators of the immune response in Drosophila (Ip et al., 1993; also see Ip and Levine, 1994). This evolutionary conservation in both structure and function of NF-κB and IκB families attests to the critical importance of this transcription factor in essential cellular processes, particularly those associated with immune system function.

Postulated Mechanism of NF-κB Activation

Most studies examining the regulation of NF-κB have focused on IκBα. Consistent with the integral role phosphoryl transfer plays in signal transduction, a widely held model explaining the mechanism of NF-κB activation stated that the inducible phosphorylation of IκBα was sufficient for its dissociation from NF-κB. This model was initially based on several studies analyzing the role of phosphorylation in vitro (Shirakawa and Mizel, 1989; Ghosh and Baltimore, 1990) and was supported by the observation that IκBα is phosphorylated in vivo following treatment

of cells with agents that activate NF-κB (Beg et al., 1993; Cordle et al., 1993; Mellits et al., 1993). IκBα also exhibits a basal level of phosphorylation that appears to be distinct from that induced by stimuli which activate NF-κB (Didonato et al., 1995; Brown et al., 1995). An additional observation of the in vivo studies was the somewhat surprising result that IκBα is rapidly degraded following the phosphorylation event (Beg et al., 1993; Brown et al., 1993; Cordle et al., 1993; Henkel et al., 1993; Mellits et al., 1993; Sun et al., 1993). The degradation of IκBα was thought to occur after its dissociation from NF-κB, a proposal supported by data demonstrating that within the cell, free IκBα is extremely labile, whereas that which is complexed to NF-κB is relatively stable (Scott et al., 1993; Sun et al., 1993; Rice and Ernst, 1993). Thus, according to this prevalent model (also see Beg and Baldwin, 1993), inducible phosphorylation causes IκBα dissociation and therefore NF-κB activation. After dissociation, uncomplexed IκBα would be rapidly degraded.

Phosphorylation of IκB Is Not Sufficient for NF-κB Activation

Although a variety of data was consistent with this popular model of NF-κB activation, two more direct experimental approaches have recently yielded results that strongly argue that the inducible phosphorylation of IκBα alone is not sufficient for NF-κB activation. First, a number of papers have shown that the phosphorylated form of IκBα induced by TNF, LPS, and other agents remains associated with NF-κB (Sun et al., 1994b; Traenckner et al., 1994; Finco et al., 1994; Miyamoto et al., 1994b; Lin et al., 1995; Didonato et al., 1995; Alkalay et al., 1995). For these experiments, NF-κB and associated proteins were immunoprecipitated from extracts of stimulated cells using antibodies specific for NF-κB. The resulting immunoprecipitate was then assayed for the presence of phosphorylated IκBα. The criterion for inducibly phosphorylated IκBα utilized in these and most other studies is its reduced mobility when analyzed by Western blot.

In the second approach, it was demonstrated that pretreatment of cells with peptide aldehydes blocked the subsequent activation of NF-κB and resulted in the accumulation of inducibly phosphorylated IκBα (Palombella et al., 1994; also see Traenckner et al., 1994; Miyamoto et al., 1994b; Lin et al., 1995; Didonato et al., 1995; Alkalay et al., 1995). Thus, under these conditions, inducibly phosphorylated IκBα is present within the cell but NF-κB is not activated. The simplest, most direct interpretation of this result is that the phosphorylated form of IκBα is still associated with NF-κB, thereby maintaining its ability to prevent NF-κB translocation into the nucleus. These results, together with those obtained by coimmunoprecipitation, convincingly argue that the inducible phosphorylation of IκBα alone is not sufficient for dissociation. Furthermore, the results obtained using peptide aldehyde protease inhibitors confirmed other observations, which suggested that an additional event required for NF-κB activation is IκB proteolysis.

Proteolysis of IκB Is Required for NF-κB Activation

Studies analyzing the role of proteolysis in NF-κB activation can be divided into two groups, depending on the type of protease inhibitor used. One group of studies utilized serine protease inhibitors, while the second group employed the previously mentioned peptide aldehydes, which are protease inhibitors that target various proteolytic activities, including calpains and the proteasome. The seminal study that indicated IκB degradation was essential for NF-κB activation came from the use of serine protease inhibitors. Pretreatment of cells with any of several serine protease inhibitors blocked the degradation of IκBα and correspondingly prevented the nuclear translocation of NF-κB (Henkel et al., 1993). Since then, others using the same protease inhibitors have reported similar results (Mellits et al., 1993; Finco et al., 1994; Mackman, 1994; Miyamoto et al., 1994a; Machleidt et al., 1994). Other forms of IκB that are targeted in the activation of NF-κB, specifically p105 and IκBβ, were also effected by the serine protease inhibitors (Mellits et al., 1993; Thompson et al., 1995). Together, these results suggested that the proteolysis of various forms of IκB was required for NF-κB activation, a conclusion subsequently confirmed through the use of peptide aldehyde protease inhibitors. Importantly, the studies using peptide aldehydes also pointed to the proteasome as the proteolytic activity within the cell that degrades IκB. However, prior to the discussion of data concerning the proteasome and NF-κB activation, a brief summary of important observations made in further studies employing serine protease inhibitors is warranted.

Serine Protease Inhibitors Prevent the Inducible Phosphorylation of IκBα

Additional studies employing serine protease inhibitors showed that they have significant pleiotropic effects on NF-κB. Some of the serine protease inhibitors can directly modify NF-κB and inhibit its DNA binding activity (Finco et al., 1994). In addition, and perhaps of more importance, all of the serine protease inhibitors examined block the inducible phosphorylation of IκBα and p105 (Mellits et al., 1993; Sun et al., 1994b; Finco et al., 1994; Miyamoto et al., 1994b; Didonato et al., 1995; Alkalay et al., 1995). These results raise a number of critical issues. First, the ability of some serine protease inhibitors to inhibit NF-κB DNA binding directly indicates that they in particular may not be ideal reagents for studies on this transcription factor. Second, the observation that serine protease inhibitors block inducible phosphorylation of IκBα suggests that these protease inhibitors may actually prevent NF-κB activation through their inhibition of IκBα phosphorylation and not its degradation. This result also raises the intriguing question as to how all of these mechanistically distinct serine protease inhibitors prevent inducible phosphorylation of IκBα. One possibility is that these protease inhibitors are targeting an additional upstream serine protease, which directly or indirectly regulates the kinase that phosphorylates IκBα. Unlike serine protease inhibitors, peptide aldehydes do not inhibit NF-κB DNA binding, nor do they prevent inducible phosphorylation of IκBα.

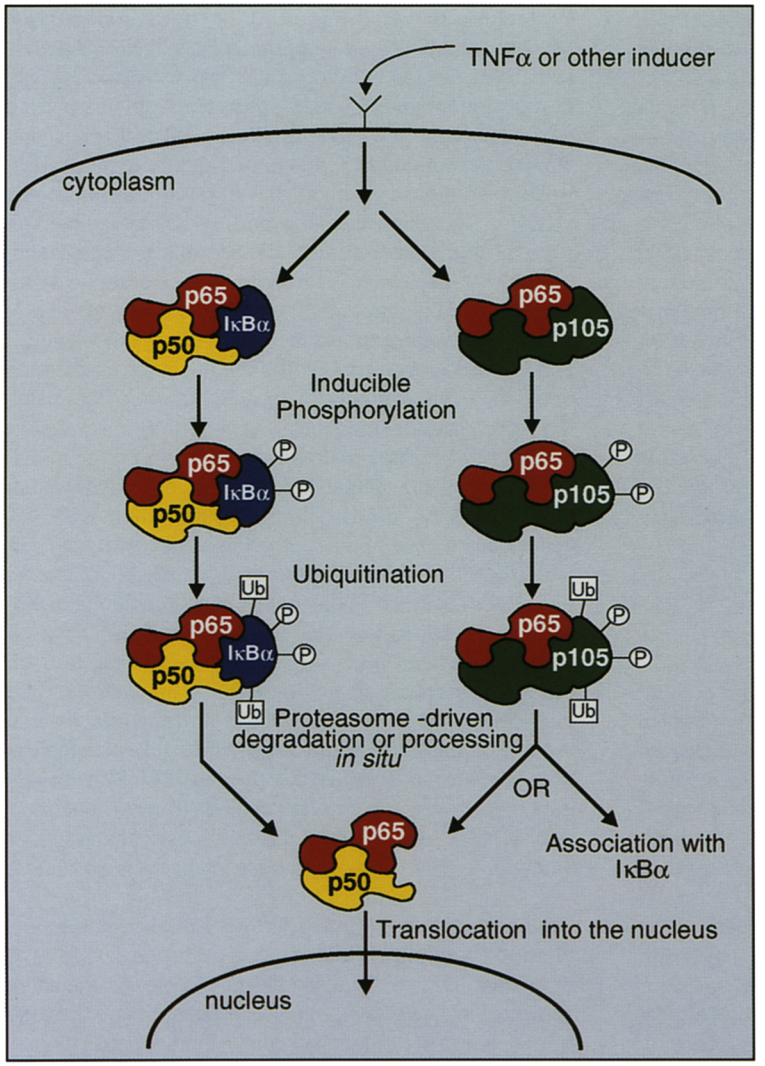

Figure 2. A Model for the Mechanism of NF-κB Activation

Inducible phosphorylation of IκBα and p105 targets these molecules for ubiquitination and subsequent proteolysis in situ by the proteasome. Liberated NF-κB then translocates into the nucleus, where it modulates gene expression. Although not shown here, inducible degradation of IκBβ may occur by a similar mechanism. Note that basal levels of phosphorylation on either NF-κB subunits or the various forms of IκB are not depicted. Refer to the text for details.

The Proteasome Is the Proteolytic Activity Responsible for IκB Degradation

Since one prominent target of peptide aldehydes is the proteasome, it was conceivable that this proteolytic complex was responsible for IκB degradation. The proteasome is a large multisubunit complex found in both the cytoplasm and nucleus. The ATP-dependent 26S (1500 kDa) proteasome complex completely degrades certain ubiquitinated and nonubiquitinated substrates, including short-lived, long-lived, and abnormal proteins (for review, see Ciechanover, 1994). By varying the type and concentration of peptide aldehyde added to cells, it is possible to distinguish whether the effects of these agents are due to their inhibition of the proteasome or instead other known protease targets, such as the calpains (Rock et al., 1994). Utilizing this information, and by analyzing the effects of other protease inhibitors, a number of studies postulated that the proteasome may be responsible for IκBα degradation during NF-κB activation (Palombella et al., 1994; Traenckner et al., 1994; Alkalay et al., 1995; Didonato et al., 1995; Lin et al., 1995). A more recent paper provides substanial evidence that supports and significantly extends this conjecture (Chen et al., 1995). This revealing study demonstrated that IκBα is ubiquitinated in vivo following treatment of cells with inducers of NF-κB and that the ubiquitinated form is degraded by the 26S proteasome. Importantly, ubiquitinated IκBα remains associated with NF-κB, strongly supporting the idea that IκBα degradation occurs while still complexed to NF-κB (i.e., in situ).

Further evidence demonstrating proteasome involvement in NF-κB activation comes from a detailed study of p105 processing (Palombella et al., 1994). This research revealed that p105 is processed to a form similar to p50 in vitro by an ATP-dependent mechanism that requires both ubiquitination and the presence of proteasomes. In addition, mutant yeast strains that are defective in proteasome subunits are incapable of processing p105 to p50. Furthermore, the peptide aldehyde protease inhibitors that are known to inhibit proteasome function also block p105 processing both in vitro and in vivo. One remarkable feature of p105 processing by the proteasome is that of controlled proteolysis, resulting in the degradation of only a portion of the p105 protein. This suggests a novel mechanism by which the proteasome is able to modulate pre-

cisely the extent to which it may degrade a protein substrate. In summary, these studies together indicate that IκB proteolysis is necessary for NF-κB activation and suggest that the proteolytic activity within the cell that is responsible for the degradation or processing of various forms of IκB is the proteasome.

A Unifying Mechanism of NF-κB Activation: Phosphorylation of IκB Signals Ubiquitination and Subsequent Degradation by the Proteasome

Since the inducible phosphorylation of IκBα does not result in its dissociation from NF-κB, what, if any, is the function of this posttranslational modification? One possibility was that the phosphorylation of IκBα targets it for ubiquitination and that the ubiquitinated form is then degraded by the proteasome in situ (see Figure 2). Recent papers provide compelling evidence that supports this model. Two serine residues in the amino terminus of IκBα have been identified as potential phospho-acceptor sites during NF-κB activation (Brown et al., 1995; Brockman et al., 1995; Traenckner et al., 1995). As one would expect, mutation of these serines to glycines or alanines prevented inducible phosphorylation, as indicated by the elimination of the characteristic shift in IκBα mobility detected on Western blot. Significantly, mutation of these sites also prevented inducible degradation. The previously described study by Chen et al. (1995) then "closes the circle" by showing that mutation of the two serines also prevents the ubiquitination of IκBα. Thus, blocking inducible phosphorylation on IκBα eliminates ubiquitination and thereby prevents proteasome-driven degradation. The ability to control proteasome activity towards specific substrates by inducible phosphorylation may explain how a constitutively active proteolytic activity, the proteasome, can participate in inducible processes, such as the degradation of IκBα. However, it is conceivable that inducible alterations to the proteasome or the enzymes responsible for ubiquitination further enhance degradation of particular substrates, including IκBα.

It has now been shown that p105 is also inducibly phosphorylated (Mellits et al., 1993; Naumann and Scheidereit, 1994; Li et al., 1994a). It is likely that the phosphorylation of p105 facilitates recognition of this molecule by the enzymes responsible for ubiquitination, followed by its proteasome-driven processing to the p50 form of NF-κB (see Figure 2). Contrary to this suggestion, the in vitro studies by Palombella et al. (1994) imply that inducible phosphorylation of p105 may not be required for its processing. However, their studies apparently focus on p105 that is not associated with NF-κB family members. Under normal cellular conditions, p105 is typically found complexed to NF-κB proteins. An attractive explanation of this disparity is that the requirements for p105 processing (and IκBα degradation) differ depending on whether they are complexed to NF-κB family members. Phosphorylation of p105 and IκBα may be required for processing or degradation of these proteins by the proteasome when complexed to NF-κB but not when free within the cell. This would also explain the observed differential stability of free IκBα versus that complexed to NF-κB. The phosphorylation of IκBα and p105 may cause a conformational change in these proteins, exposing sequences required for recognition by ubiquitin-conjugating enzymes, the proteasome, or both. PEST sequences, which are believed to target proteins for degradation, are found in the carboxyl terminus of both IκBα and p105 and may serve this function. In agreement with this notion, deletion of the carboxyl terminus of IκBα, including the PEST region, does not alter inducible phosphorylation but does prevent degradation (Brown et al., 1995; Rodriguez et al., 1995). IκBβ also contains a PEST-like region, which could act in a similar manner (Thompson et al., 1995).

An important point concerning the model in Figure 2 is that although the proteolysis of p105, IκBα, and perhaps IκBβ may occur by similar if not identical mechanisms, some studies have indicated that the kinetics of these events differ (Mellits et al., 1993; Cordle et al., 1993; Donald et al., 1995; Thompson et al., 1995). It is possible that a rate-limiting step, for instance, the phosphorylation of IκBα, p105, and IκBβ, is regulated by distinct kinases with different rates of activation. This implies that the signal transduction pathway leading to NF-κB activation may bifurcate upstream of IκB. However, it is also possible that alternative relative conformations for p105, IκBα, and IκBβ may affect the rate at which these molecules are recognized by the enzymes responsible for phosphorylation, ubiquitination, and/or proteolysis.

Reestablishment of Inactive NF-κB–IκB Complexes in the Cytoplasm

As briefly discussed earlier, NF-κB activation is typically transient. After a period of stimulation, new inactive NF-κB–IκB complexes can be detected in the cytoplasm with an accompanying loss of active NF-κB in the nucleus. Analysis of IκBα protein levels following treatment of cells with different inducers showed that after its initial loss, IκBα begins to reaccumulate in the cytoplasm (Beg et al., 1993; Brown et al., 1993; Sun et al., 1993; Henkel et al., 1993). The reappearance of IκBα protein was mirrored by an increase in its mRNA and was inhibited by pretreating cells with protein synthesis inhibitors (Beg et al., 1993; Brown et al., 1993; Sun et al., 1993; Henkel et al., 1993; Chiao et al., 1994). Therefore, it appears that a consequence of NF-κB activation is the enhanced production of IκBα protein. One exciting possibility was that this increase is the result of NF-κB-mediated expression of the IκBα gene. Data from a number of important studies have demonstrated the presence of this intriguing mechanism of negative autoregulation. First, IκBα gene expression is potently up-regulated by all inducers of NF-κB analyzed. Second, the transfection of NF-κB subunits into cells activates transcription of the endogenous IκBα gene (Brown et al., 1993; Sun et al., 1993; Scott et al., 1993). Finally, the promoter immediately upstream of the IκBα gene contains a number of near-perfect NF-κB binding sites (de Martin et al., 1993; Le Bail et al., 1993; Chiao et al., 1994; Ito et al., 1994). These sites bind NF-κB in vitro and are essential for reporter gene expression when driven by the IκBα promoter. The promoter regions of the genes encoding p105 and p100 also contain NF-κB binding sites and

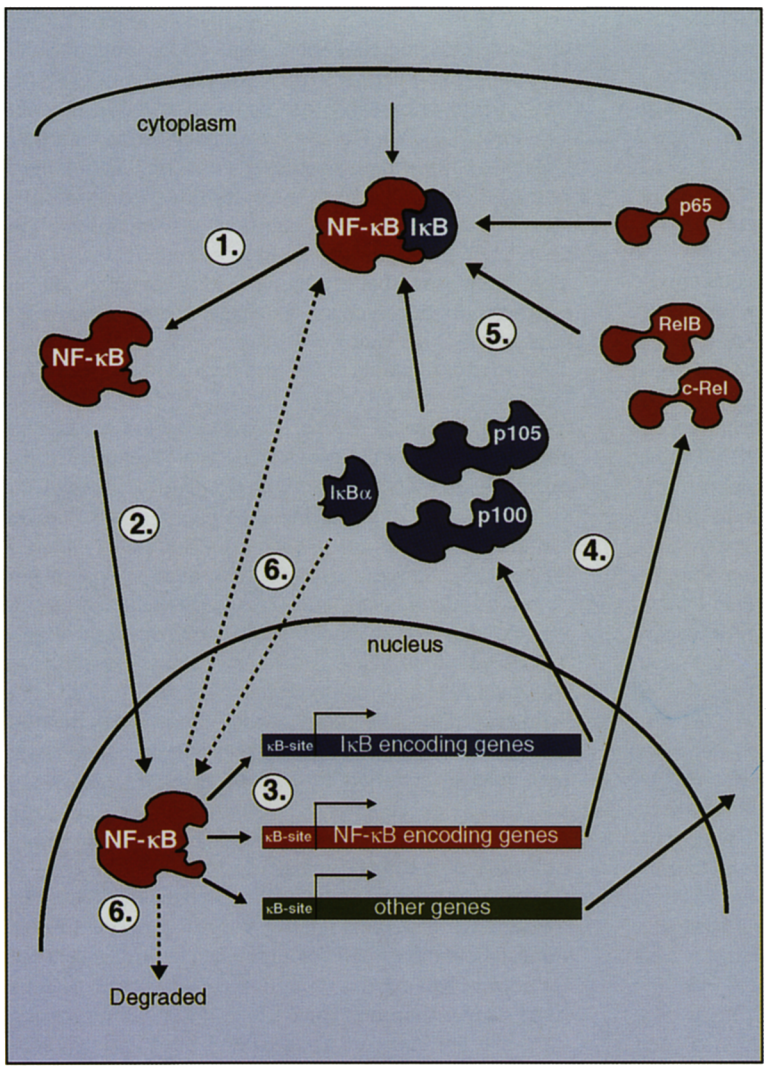

Figure 3. Mechanisms that Contribute to the Transient Activation of Transcription Factor NF-κB

(1) Treatment of cells with any of a variety of NF-κB inducers results in the proteolysis of IκB.
(2) Liberated NF-κB is translocated into the nucleus.
(3) In the nucleus, NF-κB activates a variety of genes, including those encoding IκB and NF-κB subunits.
(4) Activation of IκB and NF-κB genes results in the appearance of IκB and NF-κB protein in the cytoplasm.
(5) IκB and NF-κB complexes associate to form new NF-κB–IκB complexes, which are poised for the next round of activation.
(6) Removal of transcriptionally engaged NF-κB complexes from the nucleus is dependent on protein synthesis and likely involves the diffusion of free IκBα into the nucleus. IκBα can actively remove NF-κB from the DNA, after which the complex either retro-translocates into the cytoplasm or is perhaps degraded. In addition, uncomplexed NF-κB may be degraded by a nuclear protease. Refer to the text for details.

these genes are regulated in a manner similar to IκBα (Bours et al., 1990; Meyer et al., 1991; Mercurio et al., 1992; Ten et al., 1992; Cogswell et al., 1993; Liptay et al., 1994; Sun et al., 1994a). Moreover, the transcription of genes encoding NF-κB subunits, such as c-Rel and RelB, also appear to be activated by NF-κB (Bull et al., 1989; Hannink and Temin, 1990; Ryseck et al., 1992). Although p65 gene expression is not regulated by stimuli that activate NF-κB and its promoter lacks NF-κB binding sites, the gene is constitutively expressed at a significant basal level (Ruben et al., 1991; Ueberla et al., 1993). From this data, it appears that the reestablishment of NF-κB–IκB complexes in the cytoplasm following activation of NF-κB is in part a result of de novo protein synthesis of various forms of IκB and NF-κB (see Figure 3). The ability of NF-κB to stimulate expression of various forms of its inhibitor is an efficient means of maintaining a transient nature of activation.

The other aspect of control in the transient activation of NF-κB is the loss of transcriptionally active NF-κB from the nucleus. NF-κB typically resides in the nucleus for a limited period of time. For example, the removal from cells of stimuli such as TNF results in a steady reduction of nuclear NF-κB (Hohmann et al., 1991; Brown et al., 1993; Arenzana-Seisdedos et al., 1995). Experimental data indicates that two distinct mechanisms are responsible for this decline. The first is an inducible active process involving a newly synthesized protein, which is most likely IκBα, and the second, a more constitutive system where nuclear NF-κB is degraded, possibly by normal protein turnover. The suggestion that IκBα may down-regulate NF-κB activity in the nucleus was initially based on the observation that IκBα can directly disengage NF-κB from DNA (Zabel and Baeuerle, 1990). It was proposed that newly synthesized IκBα enters the nucleus, binds to, and removes NF-κB from the DNA. Then, the NF-κB–IκBα complexes either retro-translocate into the cytoplasm or are instead degraded within the nucleus (see Figure 3). A variety of studies have presented evidence consistent with this model. First, loss of NF-κB from the nucleus is severely curtailed when protein synthesis is inhibited, presumably because IκBα protein is not synthesized (Chiao et al., 1994; Sun

et al., 1993; Arenzana-Seisdedos et al., 1995). Second, overexpression of IκBα results in its diffusion into the nucleus (Zabel et al., 1993; Cressman and Taub, 1993). This demonstrates that IκBα can enter the nucleus when expressed at high levels, a situation which would likely exist following activation of IκBα gene expression by NF-κB. Indeed, under more physiological conditions, endogenous IκBα enters the nucleus following its synthesis in response to NF-κB inducers (Arenzana-Seisdedos et al., 1995). Importantly, the appearance of IκBα in the nucleus and its subsequent departure correlated temporally with the loss of nuclear NF-κB DNA binding. In addition, and consistent with the proposed model, a direct interaction between IκBα and NF-κB could be detected within the nucleus.

In the absence of NF-κB removal by IκBα, the transcription factor may be degraded. This is suggested by the observation that nuclear NF-κB activity eventually diminishes even in the presence of protein synthesis inhibitors (Chiao et al., 1994; Cressman and Taub, 1994) and also by a study proposing that proteolysis can contribute to the turnover of nuclear NF-κB (Cressman and Taub, 1994). The picture that emerges is one where the removal of NF-κB from the nucleus is typically an active process that requires protein synthesis and likely involves IκBα. Concurrent with or in the absence of this mechanism, nuclear NF-κB may be degraded by a nuclear protease.

Transient Versus Constitutive or Persistent Activation

Although NF-κB activation is typically transient, situations do exist where members of this transcription factor family are activated for prolonged periods. The two examples summarized below illustrate how cells, under necessary physiological conditions, are able to override the established mechanisms that favor only transient activation. The first example focuses on constitutively active forms of NF-κB found in mature B lymphocytes (Sen and Baltimore, 1986). In contrast with most cell types, mature B cells degrade IκBα at a rate that exceeds its synthesis, resulting in the constitutive nuclear translocation of a small but significant amount of NF-κB (Rice and Ernst, 1993; Miyamoto et al., 1994a; Liou et al., 1994). Thus, one means by which cells can overcome transient activation of NF-κB is to stimulate pathways responsible for IκBα proteolysis, possibly through the up-regulation of signal-transducing molecules. The rate of IκBα synthesis versus degradation may therefore influence whether NF-κB is activated transiently or constitutively. The second example pertains to certain inducers such as LPS and IL-1, which cause a persistent activation of NF-κB (Thompson et al., 1995). As shown in previous studies, treatment of cells with either TNF, IL-1, PMA, or LPS resulted in the rapid degradation of IκBα concomitant with NF-κB translocation into the nucleus. The intriguing observation of this study was that persistent inducers (LPS, IL-1) but not transient ones (PMA, TNF) also targeted IκBβ. Treatment of cells with LPS or IL-1 resulted in the degradation of IκBβ, albeit at slower kinetics than for IκBα. Importantly, IκBβ protein did not reaccumulate within the cytoplasm following activation, a result consistent with the inability of NF-κB inducers to increase IκBβ RNA levels. Apparently, only after removal of the inducer would IκBβ protein levels be reestablished. The authors propose that the rapid degradation of IκBα in response to various inducers releases NF-κB complexes involved in the initial transient activation while the slower sustained down-regulation of IκBβ yields NF-κB, which persistently resides in the nucleus. If this model is correct, a mechanism must exist that prevents newly synthesized IκBα from binding to and sequestering NF-κB released from IκBβ. Nonetheless, these results suggest that another means by which cells overcome the transient nature of NF-κB activation is by targeting forms of IκB other than IκBα.

Concluding Remarks

The existence of multiple forms of IκB in mammalian cells suggests alternative ways of regulating NF-κB activity, thereby allowing members of this transcription factor family to participate in a variety of cellular events. The proposed roles of IκBα and IκBβ in transient and persistent activation, respectively, demonstrates the relevance of this postulation. It will be important to define further how each form of IκB contributes to the regulation of NF-κB during different cellular responses.

Clearly, for NF-κB regulation to be sufficiently understood, the detailed elucidation of signal transduction pathways that target NF-κB–IκB complexes is crucial. At present, only general considerations can be made. The ability of such a large number of distinct biological agents to activate NF-κB suggests that multiple pathways may result in IκB removal and therefore NF-κB activation. In accordance with this view, an increasing number of divergent signal-transducing molecules are being implicated in NF-κB activation. Conversely, other research has indicated that most if not all inducers of NF-κB utilize a common redox-sensitive step in the activation of NF-κB (Siebenlist et al., 1994; Baeuerle and Henkel, 1994). Perhaps initially distinct pathways converge on a common pathway, which then targets individual NF-κB–IκB complexes. Another topic concerning signal transduction and the different IκB molecules is whether the various IκBs are targeted by the same pathway? As discussed earlier, it appears that similar mechanisms, i.e., phosphorylation-induced proteolysis, regulate the activity of many forms of IκB. In addition, inducible degradation of IκBα, IκBβ, and p105 is sensitive to pretreatment of cells with anti-oxidants or the serine protease inhibitor and alkylating agent TPCK. These observations suggest that highly similar or identical pathways target each of the different IκB molecules. However, the kinetics by which various types of IκB are phosphorylated and degraded differ. Furthermore, some inducers only target one form of IκB, while others target multiple forms, implying that alternative signaling pathways exist. Further research should clarify this issue.

Much of the research on NF-κB regulation has concentrated on inducible modifications to IκB. However, recent studies have shown that NF-κB is also phosphorylated in an inducible manner (Neumann et al., 1992; Druker et al., 1994; Naumann and Scheidereit, 1994; Li et al., 1994a; Diehl et al., 1995). Some studies have indicated that phos-

phorylation of NF-κB family members is important in regulating its DNA binding capacity (Hayashi et al., 1993; Naumann and Scheidereit, 1994; Li et al., 1994b). It is anticipated that modifications to NF-κB, including phosphorylation, are also important for other functions, possibly contributing to the removal of IκB, translocation of NF-κB into the nucleus, and enhancement of NF-κB transactivation capabilities.

In summary, this review has discussed the mechanisms by which cells regulate members of the NF-κB transcription factor family. The general theme that emerges from these studies is that the exquisite control of NF-κB function is cummulative in nature, requiring the coordinated phosphorylation and proteolysis of both NF-κB and IκB proteins.

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