

# Missing Pieces in the NF- $\kappa$ B Puzzle

## Review

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**The regulation of the transcription factor NF- $\kappa$ B activity occurs at several levels including controlled cytoplasmic-nuclear shuttling and modulation of its transcriptional activity. A critical component in NF- $\kappa$ B regulation is the I $\kappa$ B kinase (IKK) complex. This review is focused on recent progress as well as unanswered questions regarding the regulation and function of NF- $\kappa$ B and IKK.**

### Introduction

The recognition of pathogens by innate or adaptive immune receptors leads to activation of cells displaying these receptors, e.g., macrophages, dendritic cells, and lymphocytes. The signal generated by the liganded receptor is communicated to changes in gene expression leading to enhanced expression of effector molecules such as cytokines and adhesion molecules (Ghosh et al., 1998; Zhang and Ghosh, 2001). This process depends on activation of various inducible transcription factors, among which the NF- $\kappa$ B transcription factors play an evolutionarily conserved and critical role in the triggering and coordination of both innate and adaptive immune responses. The wide variety of genes regulated by NF- $\kappa$ B include those encoding cytokines (e.g., IL-1, IL-2, IL-6, IL-12, TNF- $\alpha$ , LT $\alpha$ , LT $\beta$ , and GM-CSF), chemokines (e.g., IL-8, MIP-1 $\alpha$ , MCP1, RANTES, and eotaxin), adhesion molecules (e.g., ICAM, VCAM, and E-selectin), acute phase proteins (e.g., SAA), and inducible effector enzymes (e.g., iNOS and COX-2). In addition, it has been demonstrated recently that genes encoding evolutionarily conserved antimicrobial peptides, such as  $\beta$  defensins, is also regulated by NF- $\kappa$ B (Ghosh et al., 1998; Zhang and Ghosh, 2001). NF- $\kappa$ B also plays a role in expression of genes encoding molecules important for the adaptive immune response, such as MHC proteins, costimulatory molecules such as B7.1, and cytokines such as IL-2, IL-12, and IFN- $\beta$  (Kopp and Medzhitov, 1999). Additionally, some of the chemokines and cytokines produced in response to NF- $\kappa$ B activation can

stimulate the migration and maturation of lymphocytes. Furthermore, NF- $\kappa$ B is central for the overall immune response through its ability to activate genes coding for regulators of apoptosis and cell proliferation, such as c-IAP-1, c-IAP-2, A1 (Bfl1), Bcl-X<sub>L</sub>, Fas ligand, c-myc, and cyclin D1, that are critical for apoptotic processes (Karin and Lin, 2002).

This long list of functions suggests that modulation of NF- $\kappa$ B activity and action should represent effective therapeutic strategies for combating diseases such as arthritis, asthma, or autoimmunity that result from hyperactivation of otherwise beneficial immune responses. Therefore, there is intense interest in understanding the regulation of this transcription factor in the context of various diseases. A number of recent reviews have described in detail various facets of NF- $\kappa$ B regulation and function (Baldwin, 2001; Ben-Neriah, 2002; Karin and Ben-Neriah, 2000; Silverman and Maniatis, 2001). To avoid duplicating these reviews, following a brief discussion of the NF- $\kappa$ B pathway, we will focus on four areas of NF- $\kappa$ B regulation that represent some of the most intriguing and outstanding questions that are likely to engage researchers in the near future.

### NF- $\kappa$ B Signaling Pathways

NF- $\kappa$ B represents a group of structurally related and evolutionarily conserved proteins, with five members in mammals: Rel (c-Rel), RelA (p65), RelB, NF- $\kappa$ B1 (p50 and its precursor p105), and NF- $\kappa$ B2 (p52 and its precursor p100) (reviewed in Ghosh et al., 1998). The mechanisms by which the p105 and p100 precursor proteins are processed are not fully understood and were suggested to involve either posttranslational or cotranslational events (Karin and Ben-Neriah, 2000). What seems to be clear is that while p105 processing is constitutive, the processing of p100 is regulated (Xiao et al., 2001). NF- $\kappa$ B/Rel proteins can exist as homo- or heterodimers, and although most NF- $\kappa$ B dimers are activators of transcription, the p50/p50 and p52/p52 homodimers can repress the transcription of their target genes (Zhong et al., 2002). NF- $\kappa$ B/Rel proteins share a highly conserved 300 amino acid long N-terminal Rel homology domain (RHD) responsible for DNA binding, dimerization, and association with the I $\kappa$ B inhibitory proteins (Ghosh et al., 1998). In resting cells, most NF- $\kappa$ B/Rel dimers are bound to I $\kappa$ Bs and retained in the cytoplasm. The I $\kappa$ Bs are also members of a gene family that contains seven known mammalian members, I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , I $\kappa$ B $\epsilon$ , I $\kappa$ B $\gamma$ , Bcl-3, and the precursor Rel proteins p100 and p105 (Ghosh et al., 1998). Interestingly, p100 may serve both as an I $\kappa$ B and as a specific heterodimeric partner for RelB, such that its processing results in the release of p50:RelB heterodimers (Solan et al., 2002). The I $\kappa$ Bs are characterized by the presence of multiple ankyrin repeats, which are protein-protein interaction domains that interact with NF- $\kappa$ B via the RHD (Ghosh et al., 1998). Nearly all NF- $\kappa$ B proteins have been crystallized, and their structures, as well as those of cocrystals of NF- $\kappa$ B with I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$ , have been determined (Chen and Ghosh, 1999; Chen et

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al., 1998a, 1998b; Ghosh et al., 1995; Huang et al., 2001; Huxford et al., 1998; Jacobs and Harrison, 1998; Muller et al., 1995). These structural studies revealed that the RHD forms a unique butterfly-shaped structure that is composed of  $\beta$  strands arranged in a pattern similar to immunoglobulin domains. The structure of the  $I\kappa B\alpha$ :NF- $\kappa B$  complexes reveal a characteristic structure for the  $I\kappa B$  ankyrin repeats, where each repeat consists of two closely packed helices followed by a loop and a tight hairpin turn (Huxford et al., 1998; Jacobs and Harrison, 1998). The multiple repeats stack to form a slightly curved cylinder with the loops forming finger-like extensions. Interestingly, the dimerization domain of the NF- $\kappa B$  dimers is the primary region of interaction with  $I\kappa B$ s, although critical differences in the nature of the interactions mounted by  $I\kappa B\alpha$  and  $I\kappa B\beta$  play crucial roles in the regulation of NF- $\kappa B$  activity (G. Ghosh, personal communication).

Cell stimulation with a variety of agonists triggers signal transduction pathways that ultimately result in activation of a specific  $I\kappa B$  kinase (IKK) (Karin and Ben-Neriah, 2000). IKK is a complex composed of three subunits:  $IKK\alpha$  ( $IKK1$ ),  $IKK\beta$  ( $IKK2$ ), and  $IKK\gamma$  (NEMO,  $IKKAP$ ) (Karin and Ben-Neriah, 2000).  $IKK\alpha$  and  $IKK\beta$  are the catalytic subunits of the complex, sharing 52% overall sequence identity and 65% identity in their catalytic domains. The third subunit,  $IKK\gamma$ /NEMO, is the regulatory subunit and is not related to the catalytic subunits (Rothwarf and Karin, 1999). Gel filtration analysis indicates that IKK is a large complex, 700–900 kDa in size, suggesting the presence of additional components. Recently, Cdc37 and Hsp90 were suggested to serve as additional components of the IKK complex (Chen et al., 2002). The functional importance of these proteins to IKK was suggested by treating cells with geldanamycin (GA), an antitumor agent that interferes with the function of Hsp90. Such treatment reduced the size of the IKK complex and abolished TNF-induced activation of IKK and NF- $\kappa B$ . However, GA was not reported to inhibit other modes of IKK activation, and Hsp90 is known to be associated with many protein kinases (Fisher et al., 2000; Goes and Martin, 2001; Sato et al., 2000). In another study, geldanamycin was shown to cause the degradation of another kinase associated with Hsp90 called RIP (Lewis et al., 2000). Knockout studies have shown that RIP is essential for TNF $\alpha$ -induced NF- $\kappa B$  activation (Kelliher et al., 1998). Thus, it is not clear whether Hsp90 is a functional component of IKK or simply associated with many kinases, some of which happen to be required for TNF $\alpha$  signaling. In vitro,  $IKK\alpha$  and  $IKK\beta$  exhibit similar substrate specificities, targeting two specific serines in the N-terminal regulatory domain of  $I\kappa B$  proteins (Zandi et al., 1997). However,  $IKK\beta$  is a more potent  $I\kappa B$  kinase than  $IKK\alpha$ . By and large, the same major stimuli that activate NF- $\kappa B$ , including byproducts of microbial, fungal, and viral infections and proinflammatory cytokines, also activate IKK, and thus, as we shall discuss below, IKK activity is absolutely essential for NF- $\kappa B$  activation.

Phosphorylation of  $I\kappa B$ s by IKK tags them for polyubiquitination by a specific ubiquitin ligase belonging to the SCF (Skp-1/Cul/F box) family (Ben-Neriah, 2002). The actual recognition of N-terminally phosphorylated  $I\kappa B$ s is carried out by a WD repeat- and F box-containing protein called  $\beta$ -TrCP (Ben-Neriah, 2002). Upon ubiquiti-

nation, the  $I\kappa B$  proteins are rapidly degraded by the proteasome, thereby freeing NF- $\kappa B$ , which then enters the nucleus, binds to DNA, and activates transcription. As we will discuss later, it now appears that nuclear NF- $\kappa B$  must also become posttranslationally modified to be transcriptionally active, although the exact nature of the modifications involved remains to be fully elucidated. IKK is also involved in phosphorylation-induced processing of p100, resulting in the activation of p52:RelB dimers (Senftleben et al., 2001a). The biochemical events that follow p100 phosphorylation, resulting in its processing, are yet to be described.

### Cytoplasmic to Nuclear Transport of NF- $\kappa B$ Molecules: Is This Paradigm Really True?

The defining characteristic of NF- $\kappa B$  is its rapid inducibility, which allows cells to respond to infectious organisms and stress by upregulating appropriate NF- $\kappa B$  target genes. Since the original classical experiment of Baeuerle and Baltimore, who used a detergent to unmask the DNA binding activity of NF- $\kappa B$  in the cytosol of unstimulated cells, the central paradigm of NF- $\kappa B$  regulation has been the translocation of activated NF- $\kappa B$  from the cytoplasm to the nucleus (Baeuerle and Baltimore, 1988). The basis for the cytoplasmic localization of the inactive NF- $\kappa B$ : $I\kappa B$  complex is thought to be due to masking of the nuclear localizing signals (NLS) on the NF- $\kappa B$  subunits by the  $I\kappa B$  proteins. Thus,  $I\kappa B$  degradation would simply lead to unmasking of the NLS, allowing free NF- $\kappa B$  dimers to enter the classical nuclear import pathway. However, a number of recent studies question the accuracy or generality of this simple model.

The first set of such reports demonstrated that following NF- $\kappa B$  activation, newly synthesized  $I\kappa B\alpha$  molecules enter the nucleus and remove NF- $\kappa B$  from DNA (Arenzana-Seisdedos et al., 1995; Brown et al., 1993; Chiao et al., 1994; Sun et al., 1993). The discovery that  $I\kappa B\alpha$  contained leucine-rich nuclear-export sequences (NES) supported the idea that newly synthesized  $I\kappa B\alpha$  transports NF- $\kappa B$  back to the cytoplasm (Arenzana-Seisdedos et al., 1997; Rodriguez et al., 1999). However, an NES was not found in  $I\kappa B\beta$  (Malek et al., 2001; Tam and Sen, 2001), which through gene replacement experiments was shown to be functionally equivalent to  $I\kappa B\alpha$  (Cheng et al., 1998). Furthermore, the mechanism responsible for nuclear uptake of  $I\kappa B\alpha$  remained controversial. One study suggested the action of a nonclassical NLS within the second ankyrin repeat of  $I\kappa B\alpha$  (Sachdev et al., 1998), whereas another report suggested a “piggyback” mechanism through which  $I\kappa B\alpha$  used the NLS of an associated protein (Turpin et al., 1999). A more likely explanation for the nuclear uptake of NF- $\kappa B$ : $I\kappa B\alpha$  complexes is the incomplete masking of the p50 NLS as described below (Malek et al., 2001). The story became more complex when CRM1, a key mediator of nuclear export, and leptomycin B (LMB), a specific CRM1 inhibitor, were identified (Ossareh-Nazari et al., 1997). Although the NES found in  $I\kappa B\alpha$  was located near its C terminus, subsequent analysis suggested that a divergent NES located near the N terminus (between amino acids 45 and 55) was the functional motif (Huang et al., 2000; Johnson et al., 1999; Tam et al., 2000). Remarkably, treatment of unstimulated cells with LMB resulted

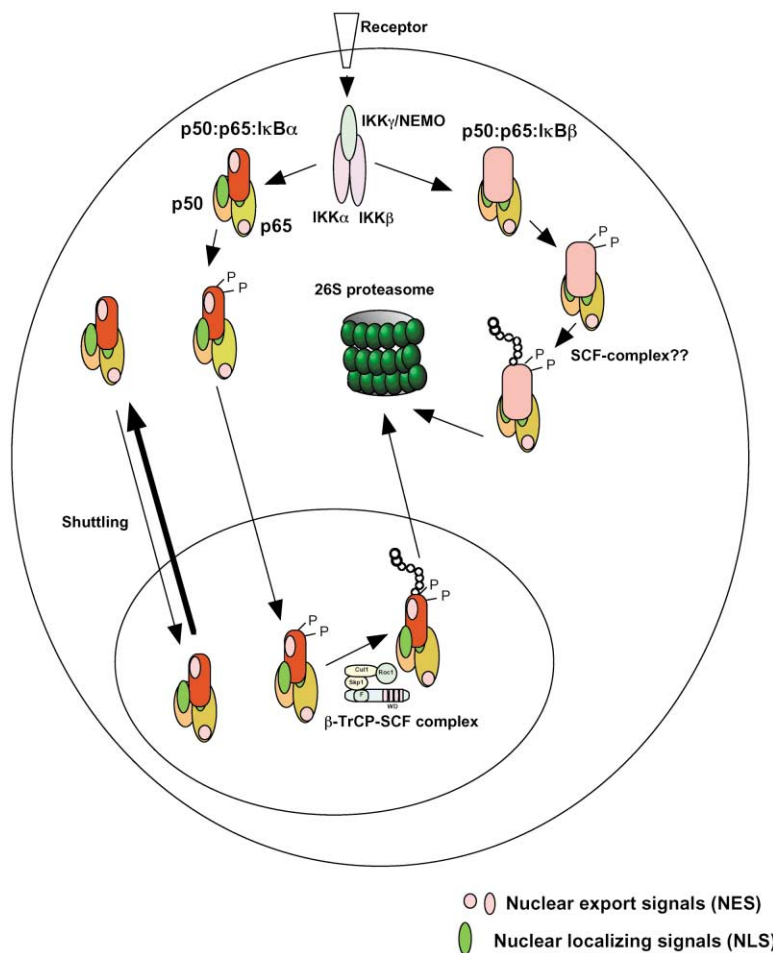


Figure 1. A Model Depicting the Differential Regulation of IκBα and IκBβ Complexes

IκBα-containing complexes appear to continuously shuttle in and out of the nucleus although their default location is the cytosol. IκBβ complexes are exclusively cytosolic.

in almost complete redistribution of NF-κB:IκBα complexes to the nucleus, suggesting that these complexes continuously shuttle in and out of the nucleus (Huang et al., 2000; Johnson et al., 1999; Rodriguez et al., 1999; Tam et al., 2000). However, the default location for this complex is the cytoplasm because the effect of the NES is dominant over that of the NLS (Figure 1). Moreover, nuclear export of NF-κB:IκB complexes may be enhanced by the presence of an additional NES on p65 (Harhaj and Sun, 1999). Although the existence of a potential NLS on IκBα itself remains to be validated, the crystallographic structure of a p50:p65:IκBα complex provides a possible explanation for the nuclear entry of these complexes (Huxford et al., 1998; Jacobs and Harrison, 1998; Malek et al., 2001). This structure reveals that IκBα can mask only the NLS on p65, while the NLS of p50 remains exposed. It is not surprising, therefore, that the NF-κB:IκBα complex efficiently enters the nucleus but is immediately expelled as a result of potent NES signals on IκBα and p65.

If the NF-κB:IκBα complex indeed shuttles in and out of the nucleus, the paradigm of cytoplasm to nuclear translocation would still be maintained if the steps leading to IκB phosphorylation and degradation occurred exclusively in the cytosol. Several findings support this notion. First, the IKK complex is almost exclusively cytoplasmic, with only small amounts of IKKα present in the

nucleus (M.K., unpublished observations). Second, IκBα degradation is blocked in LMB-treated cells, where the NF-κB:IκBα complex was exclusively nuclear (Rodriguez et al., 1999). However, other studies suggest that the lack of IκBα degradation in LMB-treated cells resulted from long periods of incubation with the inhibitor, and that in cells treated for shorter times, IL-1 and TNF stimulation induced the degradation of nuclear IκBα (Renard et al., 2000). While this discrepancy remains to be resolved, additional support for nuclear degradation of IκBα was obtained from a different source. As described above, phosphorylated IκBα is recognized by β-TrCP. This receptor protein targets the phosphorylated IκBα for ubiquitination by coupling with the Skp1-Cul-Roc ubiquitin ligase complex (Ben-Neriah, 2002). Although IκBα phosphorylation is assumed to occur in the cytosol, β-TrCP is quite unexpectedly exclusively localized in the nucleus (Figure 1; Davis et al., 2002). The nuclear localization of β-TrCP appears to be via its association with hnRNP-U, an abundant ribonucleoprotein that may serve as a pseudosubstrate for the ubiquitin ligase. Most likely, the interaction between β-TrCP and hnRNP-U is out-competed by phosphorylated IκBα, suggesting that IκBα ubiquitination takes place in the nucleus before undergoing proteasome-mediated degradation (Davis et al., 2002). Whether degradation occurs in the nucleus via nuclear proteasomes or whether the ubiquitinated

NF- $\kappa$ B:I $\kappa$ B $\alpha$  is reexported back to the cytosol has yet to be determined.

The multiplicity of reports from numerous laboratories that demonstrate a dynamic shuttling of NF- $\kappa$ B:I $\kappa$ B $\alpha$  complexes provides certain confidence that at least some aspects of the model are likely to be valid. However, these findings invite the more important question of why this shuttling occurs in unstimulated cells. What advantage is gained by this process in ensuring the inducible activity of NF- $\kappa$ B? While the presence of an effective NES on I $\kappa$ B $\alpha$  is fully consistent with its role in postinduction inactivation of NF- $\kappa$ B, having the nuclear NF- $\kappa$ B:I $\kappa$ B complex serve as a substrate for inducible ubiquitination and degradation seems difficult to comprehend from a biological standpoint (Figure 1). In particular, this process would appear to eliminate the regulation that is imposed by physical partitioning of the active transcription factor from the inactive cytosolic pool. It is important to note, however, that nearly all of the studies on the shuttling of NF- $\kappa$ B:I $\kappa$ B $\alpha$  complexes depend on the use of LMB and that different biochemical approaches have failed to detect nuclear NF- $\kappa$ B:I $\kappa$ B complexes in nonstimulated cells. Therefore, a valid concern is that the observed effects are due to an artifactual unmasking of the NLS or some other problems associated with the use of LMB.

Based on these studies, should we discard the original hypothesis that NF- $\kappa$ B activation requires nuclear import of free NF- $\kappa$ B following phosphorylation-induced I $\kappa$ B degradation in the cytosol? The answer is not entirely. While I $\kappa$ B $\alpha$  is the major isoform being studied by the vast majority of workers in the field, in many cells nearly half of the NF- $\kappa$ B is sequestered by the other major I $\kappa$ B isoform, I $\kappa$ B $\beta$  (Whiteside et al., 1997). The genetic experiments mentioned above indicate that I $\kappa$ B $\beta$  can fully replace I $\kappa$ B $\alpha$  (Cheng et al., 1998). I $\kappa$ B $\beta$ , however, does not possess a NES and its subcellular localization is not altered by LMB (Malek et al., 2001; Tam and Sen, 2001). Furthermore, unlike I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$  efficiently masks the NLSs on both Rel subunits of NF- $\kappa$ B complexes. Therefore, I $\kappa$ B $\beta$ -containing complexes are unable to shuttle under normal conditions since they lack a free NLS (Figure 1). The potential functional equivalence between I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  makes it difficult to assign a critical role for nuclear shuttling of NF- $\kappa$ B:I $\kappa$ B $\alpha$  complexes in the normal regulation of NF- $\kappa$ B activity. Therefore, at this stage of analysis, it would be fair to conclude that while nuclear shuttling of NF- $\kappa$ B:I $\kappa$ B $\alpha$  complexes clearly occurs, the biological significance of this process remains to be established.

#### How Important Is Regulation of Nuclear NF- $\kappa$ B Transcriptional Activity?

If the major step regulating NF- $\kappa$ B activity is removal of I $\kappa$ B from NF- $\kappa$ B:I $\kappa$ B complexes, it could be argued that the control of I $\kappa$ B degradation via IKK-mediated phosphorylation is the most important point of regulation for the entire pathway. However, the regulation of NF- $\kappa$ B activation does not rely entirely on IKK, and recent studies have indicated that the capacity of nuclear NF- $\kappa$ B to drive transcription is also a regulated process.

The first suggestion that some NF- $\kappa$ B/Rel proteins, particularly p65, are posttranslationally regulated came

from the work of Scheidereit and colleagues who demonstrated inducible phosphorylation of p65 following cellular stimulation (Naumann and Scheidereit, 1994; Neumann et al., 1995). Most Rel proteins have a consensus recognition site (RRXS) for cyclic AMP-dependent protein kinase (PKA) located around 25 amino acids upstream to their NLS within the RHD, suggesting that PKA-mediated phosphorylation of NF- $\kappa$ B might be involved in both its inducible and constitutive activation (Mosialos and Gilmore, 1993; Neumann et al., 1995). In addition, phosphorylation of recombinant p65:p65 homodimers and p65:p50 heterodimers by PKA enhanced their DNA binding activity (Mosialos and Gilmore, 1993; Neumann et al., 1995).

The importance of the PKA phosphorylation site in p65 at S276 was underscored by copurification of the PKA catalytic subunit (PKAc) with cytosolic NF- $\kappa$ B:I $\kappa$ B complexes (Zhong et al., 1997). The PKAc bound to NF- $\kappa$ B:I $\kappa$ B complexes was found to be inactive, but it could be activated following I $\kappa$ B degradation. The activated PKAc most likely phosphorylates p65 at S276 after its activation, although it was also reported that in some cell types cytosolic p65 is constitutively phosphorylated on S276, suggesting that phosphorylation might occur during assembly of the NF- $\kappa$ B-I $\kappa$ B-PKAc complex (Anrather et al., 1999). The role of this phosphorylation appears to be 2-fold. First, it is necessary for enhancing DNA binding by p65; and second, it provides an additional interaction site for the transcriptional coactivator CBP/p300 (Zhong et al., 1998, 2002). Before phosphorylation, the C-terminal region of p65 interacts with the RHD, thereby interfering with both DNA and CBP/p300 binding. Phosphorylation of S276 prevents this intramolecular association, thereby facilitating both DNA binding and transcriptional activity of NF- $\kappa$ B.

A number of recent studies lend support to the proposal that p65 phosphorylation may be necessary for transcriptional competence of nuclear NF- $\kappa$ B (Bird et al., 1997; Madrid et al., 2000, 2001; Wang et al., 2000). In addition to PKA, protein kinases such as casein kinase II, PKC $\zeta$ , and IKK itself have been implicated in this process (Leitges et al., 2001; Wang et al., 2000). The relative importance of each of these kinases in NF- $\kappa$ B activation is yet to be determined, but the conclusion from these separate studies is that posttranslational modification of p65 is critical for regulating NF- $\kappa$ B transcriptional activity. In addition, knockouts of the serine-threonine protein kinases GSK3 $\beta$  and Tbk/t2k/NAK also revealed dramatic effects on the transcriptional activity of NF- $\kappa$ B (Bonnard et al., 2000; Hoeflich et al., 2000; Tojima et al., 2000). Cells from mice lacking either kinase demonstrated normal activation of NF- $\kappa$ B in response to a wide variety of inducers when measured by I $\kappa$ B degradation, NF- $\kappa$ B nuclear translocation, or binding to DNA. But in both instances, nuclear NF- $\kappa$ B was unable to drive transcription. These results suggest that the embryonic lethality in these knockout strains caused by massive hepatocyte apoptosis, a phenotype identical to that observed in mice lacking either p65 or IKK $\beta$  (Beg et al., 1995; Li et al., 1999b, 1999d; Tanaka et al., 1999), is due to defective activation of NF- $\kappa$ B-regulated antiapoptosis genes. More recently, deletion of the NF- $\kappa$ B-inducing kinase (NIK) resulted in an unexpected effect on NF- $\kappa$ B transcriptional activity (Yin et al., 2001).

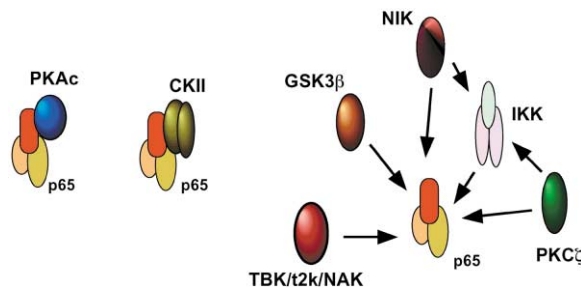


Figure 2. A Depiction of the Different Protein Kinases that Have Been Implicated in the Regulation of NF- $\kappa$ B Transcriptional Activity. Protein kinase A catalytic subunit (PKA $\alpha$ ) and casein kinase II (CKII) have been shown to be directly associated with the cytosolic NF- $\kappa$ B:IkB complex, whereas the involvement of PKC $\zeta$ , GSK3 $\beta$ , TBK1/t2k/NAK, and NIK have been deduced from knockout studies.

Unlike GSK3 $\beta^{-/-}$  and *tbk1/NAK/t2k* $^{-/-}$  mice, NIK $^{-/-}$  mice are viable but cells derived from them fail to generate transcriptionally active NF- $\kappa$ B in response to lymphotoxin  $\beta$  (LT $\beta$ ). Surprisingly, the response to many other NF- $\kappa$ B inducers is normal, suggesting NIK may play an important role in the transcriptional activation of nuclear NF- $\kappa$ B, but only in the context of LT $\beta$  signaling. While the participation of NIK in p100 processing to p52 might partially explain the lowered transcriptional activity (discussed below), the level of nuclear NF- $\kappa$ B complexes appears quite normal, suggesting that transcriptional competence of these nuclear complexes is also affected (Yin et al., 2001). Recently it was suggested that although IKK $\alpha$  is not required for IkB degradation and induction of NF- $\kappa$ B DNA binding activity, it is required for generation of transcriptionally competent NF- $\kappa$ B (Sizemore et al., 2002). As NIK is thought to act upstream to IKK $\alpha$  in the processing pathway (Senftleben et al., 2001a), it is possible that the effect of NIK on NF- $\kappa$ B transcriptional activity is mediated through IKK $\alpha$  (Figure 2). The deletion of the atypical protein kinase PKC $\zeta$  also had a significant effect on the transcriptional activity of NF- $\kappa$ B, but only in embryonic fibroblasts (Leitges et al., 2001). In other tissues, such as lung, lack of PKC $\zeta$  also affected the activation of IKK, suggesting that it might serve different roles in different tissues (Leitges et al., 2001). However, similar to NIK, the ability to PKC $\zeta$  to affect the activity of IKK raises the possibility that the effect of PKC $\zeta$  on the transcriptional activity of NF- $\kappa$ B might also be mediated through IKK (Figure 2).

Stimulation of cells by TNF $\alpha$  has also been shown to result in inducible p65 phosphorylation at position 529, which is mediated by casein kinase II (CKII) (Wang et al., 2000). The mechanism by which p65 phosphorylation by CKII is regulated is similar to that proposed for PKA. CKII is constitutively active in most cells, but its ability to phosphorylate p65 is blocked by association with IkB $\alpha$ . IkB $\alpha$  degradation relieves this repression, enabling inducible phosphorylation of p65. More recently, phosphatidylinositol-3 kinase (PI3K) and Akt were also suggested to affect the transcriptional activity of p65 in response to IL-1 stimulation (Madrid et al., 2000, 2001). However, neither PI3K nor Akt seems to directly phosphorylate p65; instead, PI3K-activated Akt activates IKK $\alpha$ , which then phosphorylates p65 at S536. Notably,

IKK has been shown in transient transfection experiments to phosphorylate p65 at S536. The one complication in this model is that direct biochemical analysis failed to reveal IKK activation by Akt (Delhase et al., 2000).

The possible involvement of so many different kinases in controlling p65 transcriptional activity seems unusual. It is not clear whether these kinases lie in a linear pathway that leads to a single phosphorylation site in p65 or whether they all target distinct sites on p65 (Figure 2). If the latter hypothesis is true, then do the different p65 kinases respond to certain inducers but not others? Also, for PKA and CKII, which are both thought to associate with NF- $\kappa$ B:IkB complexes, what fraction of the cellular pool of the latter is associated with each kinase? Finally, almost nothing is known about how GSK3 $\beta$  and TBK1/t2k/NAK might influence NF- $\kappa$ B transcriptional activity and, as mentioned above, they or other kinases may also act via other transcription factors. It seems that a crucial experiment that would at least help answer some of these questions would be to determine whether these kinases can directly phosphorylate p65 and then mutate the putative phosphoacceptor sites for these kinases. Preferably, the mutant alleles should be created in mice using a knockin strategy. Recently, a similar approach helped to establish the function of the putative activating phosphorylation sites on IKK $\alpha$  (Cao et al., 2001).

One possible explanation for the critical role of p65 phosphorylation is that it serves to recruit histone acetyltransferases (HATs) to nuclear NF- $\kappa$ B. Multiple studies implicated both CBP/p300 and p/CAF HATS in NF- $\kappa$ B regulation (Gerritsen et al., 1997; Merika et al., 1998; Perkins et al., 1997; Sheppard et al., 1999; Zhong et al., 1998), and it is therefore not surprising that recent findings have begun to demonstrate roles for histone deacetylases (HDACs) as well. It was shown that p65 can interact with distinct HDAC isoforms and that these interactions negatively regulate gene expression (Ashburner et al., 2001; Chen et al., 2001; Ito et al., 2000; Lee et al., 2000; Zhong et al., 2002). It is unclear, however, which HDAC isoform associates with p65. One report demonstrated that HDAC1 and HDAC2 (via binding to HDAC1) associate with p65, when tested by immunoprecipitation of endogenous HDACs from cells stably transfected with p65 (Ashburner et al., 2001). Two other studies reported association of endogenous HDAC1 with endogenous p65 in immunoprecipitation analysis (Chen et al., 2001; Zhong et al., 2002). In GST pull-down assays, both HDAC1 and HDAC2 were found to associate with the p65-HAT complex (Ito et al., 2000; Lee et al., 2000). Intriguingly, however, a more recent report demonstrates that TNF $\alpha$ -induced, p300-dependent acetylation of p65 is reversed by HDAC3 recruitment (Chen et al., 2001). According to this model, acetylation of p65 determines the efficiency of its interaction with IkB $\alpha$ . Therefore, the acetylated p65 that enters the nucleus is refractory to inhibition by IkB $\alpha$ , but following HDAC3 recruitment, the deacetylated p65 associates with IkB $\alpha$  and is exported to the cytoplasm, downregulating NF- $\kappa$ B activity. These findings are consistent with prior observations regarding IkB $\alpha$ -mediated downregulation of nuclear NF- $\kappa$ B and could represent a generally applicable mechanism for regulating association of proteins in the nucleus. However, the effect of HDAC3 on acetylated histones associated with promoters of p65



target genes was not examined, and the possibility that HDAC3 could be replaced by other HDAC isoforms was not directly tested (Chen et al., 2001). Therefore, it is unclear if the observed effects are specific for HDAC3. Moreover, the level of acetylated p65 was very low, suggesting that the entire complement of nuclear p65 might not be acetylated and hence, the scope of the effects of HDAC3 would be limited. Consequently, the major stimulatory effect of CBP/p300 on NF- $\kappa$ B-dependent transcription might still be through acetylation of histones or other proteins in the chromatin-remodeling machineries, and the inactivating effects of deacetylation may predominantly target acetylated chromatin components and be carried out by multiple HDAC isoforms.

The most abundant form of NF- $\kappa$ B in cells is the p50:p65 heterodimer, although low levels of other family members have been identified in the nuclei of unstimulated cells. In particular, homodimers of p50 which lack a transactivation domain can be readily detected in resting cell nuclei (Kang et al., 1992; Ten et al., 1992). Intriguingly, although p50 cannot recruit HATs directly, it can promote transcription *in vitro* via its ability to interact with other nuclear proteins, including Bcl-3, that recruit associated HATs (such as Tip60) (Dechend et al., 1999). However, several lines of evidence demonstrate that overexpression of p50 or enhanced DNA binding by p50 causes decreased expression of NF- $\kappa$ B-dependent genes (Ledebur and Parks, 1995; Plaksin et al., 1993). Furthermore, genetic defects that eliminate p50-DNA binding release such suppression and enhance gene expression (Udalova et al., 2000). It has been assumed that these repressive effects occur through competition for DNA binding with complexes containing transactivating NF- $\kappa$ B proteins such as p65. However, this is an unlikely explanation as the affinity of p50 homodimers for classical  $\kappa$ B site DNA is significantly lower than that of NF- $\kappa$ B heterodimers such as p50:p65 (Phelps et al., 2000). It therefore appears that an alternative mechanism accounts for the ability of p50 (that is capable of transactivation *in vitro*) to repress  $\kappa$ B-dependent gene expression.

One potential mechanism underlying transcriptional repression by p50 homodimers is the recruitment of corepressor complexes containing specific HDACs to the promoter regions of  $\kappa$ B-dependent genes. This hypothesis received attention recently, and it has now been shown that the complement of nuclear NF- $\kappa$ B complexes in unstimulated cells consists primarily of p50 homodimers associated with HDAC-1 (Zhong et al., 2002). The ability of such p50:HDAC-1 complexes to repress gene expression was demonstrated using the specific HDAC-inhibitor trichostatin A (TSA) that upregulated expression of a subset of  $\kappa$ B-dependent genes in wild-type but not p50<sup>-/-</sup> cells (Zhong et al., 2002). These studies also demonstrated that following cell stimulation, NF- $\kappa$ B complexes comprising p50 associated with p65 that was phosphorylated in the cytoplasm by PKAc, enter the nucleus, and displace the p50:HDAC-1 complexes from  $\kappa$ B sites within certain target gene promoters. These findings may therefore provide a reason for the added layer of regulation imposed by phosphorylation of p65, as such a process would ensure that only NF- $\kappa$ B heterodimers present in the nucleus following

cell stimulation would be transcriptionally active and capable of driving gene expression.

### Regulation of IKK Activity: How Is the IKK Complex Activated?

It can be argued that of all the different steps involved in NF- $\kappa$ B activation and modulation of its transcriptional activity, the most critical one is the translocation of NF- $\kappa$ B dimers (mostly p50:p65 and p50:c-Rel) to the nucleus. Without entering the nucleus, NF- $\kappa$ B cannot regulate transcription, and all other aspects of NF- $\kappa$ B regulation become irrelevant. Nevertheless, it is important to realize that not all NF- $\kappa$ B dimers are regulated alike. For instance, as described above, some NF- $\kappa$ B dimers are constitutively nuclear in nonstimulated cells, where they may serve as transcriptional repressors. Also, the nuclear entry of p50:p65 and p50:cRel dimers are regulated differently from that of p52:RelB dimers. In general, inducible nuclear entry is a highly regulated process requiring the coordinated action of more than three dozen different proteins (Karin and Ben-Neriah, 2000). However, with the exception of the I $\kappa$ B kinase (IKK), whose activity is highly regulated, all of these proteins are either constitutively active or lack enzymatic activity. Therefore, the understanding of NF- $\kappa$ B regulation and function is tightly linked to the understanding of IKK regulation and function.

Gene-targeting experiments have clearly demonstrated that the IKK $\beta$  and IKK $\gamma$  subunits of IKK are required for NF- $\kappa$ B activation by all known proinflammatory stimuli, including bacterial lipopolysaccharide (LPS), negative-strand RNA viruses, double-stranded (ds)RNA, immunostimulatory DNA sequences (ISS-DNA), TNF $\alpha$ , IL-1, and antigens (Chu et al., 2000; Li et al., 1999b, 1999d; Makris et al., 2000; Rudolph et al., 2000; Schmidt-Supprian et al., 2000; Senftleben et al., 2001b; Tanaka et al., 1999). All of these stimuli lead to IKK activation, whose kinetics and magnitude are directly related to those of NF- $\kappa$ B activation. However, the intriguing question of how these diverse stimuli converge to activate a single signaling enzyme remains for the most part unanswered. Although the complex mechanistic details regarding IKK activation are still being unraveled, most of the currently available results that have been verified genetically point to one general mechanism requiring IKK phosphorylation.

Both IKK $\alpha$  and IKK $\beta$  can be produced as active recombinant proteins using baculovirus or yeast expression systems (Miller and Zandi, 2001; Zandi et al., 1998). However, in mammalian cells, their activation depends on association with the IKK $\gamma$  regulatory subunit (Makris et al., 2000; Rothwarf and Karin, 1999; Yamaoka et al., 1998). The activity of IKK $\alpha$  and IKK $\beta$  also depends on their ability to dimerize via their leucine zipper motifs (Mercurio et al., 1997; Woronicz et al., 1997; Zandi et al., 1997). Once IKK $\alpha$  and IKK $\beta$  dimerize, they can associate with IKK $\gamma$  through a short interaction motif located at the very C terminus of either catalytic subunit (Hu et al., 2001; May et al., 2000). Short peptide mimics of this interaction motif can be used to disrupt the IKK complex and prevent its activation (May et al., 2000). The association of IKK $\alpha$  and IKK $\beta$  dimers (usually IKK $\alpha$ :IKK $\beta$  heterodimers) with IKK $\gamma$  results in formation of a large complex,

composed of IKK $\alpha$ :IKK $\beta$  heterodimers held together via dimeric interactions between two IKK $\gamma$  molecules (Miller and Zandi, 2001; Rothwarf and Karin, 1999). At this point, it is not clear whether different cell types contain IKK complexes of different composition of IKK $\alpha$  and IKK $\beta$  subunits. Nevertheless, complex assembly is essential for stimulus-dependent IKK activation (Rothwarf and Karin, 1999). In addition to its role in complex assembly, IKK $\gamma$  serves an important regulatory function by connecting IKK to upstream activators through its C terminus, which contains a Zn finger motif. Truncation of the IKK $\gamma$  C terminus or single amino acid substitutions within it do not affect assembly of the large IKK holoenzyme but do compromise its activation (Rothwarf and Karin, 1999). Certain IKK and NF- $\kappa$ B activators, such as the Tax protein of HTLV-1, were proposed to act by directly contacting IKK $\gamma$  (Carter et al., 2001).

Apart from assembly of this large IKK holocomplex, activation of IKK by all proinflammatory and innate immune stimuli depends on phosphorylation of either the IKK $\beta$  or IKK $\alpha$  catalytic subunits at two conserved serines located within their activation loops (Delhase et al., 1999; Ling et al., 1998; Mercurio et al., 1997). Regulation of protein kinase activity through phosphorylation of specific sites within the activation loop is a very common mechanism (Taylor et al., 1995). Such phosphorylation may be achieved either through the action of an upstream kinase or through transautophosphorylation brought about by induced proximity between two catalytic subunits within the same complex. The latter mechanism may be more evolutionarily ancient.

Cell stimulation with either TNF $\alpha$  or IL-1 rapidly increases IKK $\alpha$  and IKK $\beta$  phosphorylation (Delhase et al., 1999). In the case of IKK $\beta$ , the first sites to be phosphorylated are the activation loop serines, whose replacement with alanines generates a mutant form of IKK $\beta$  that, when incorporated into IKK holocomplexes, prevents activation in response to TNF $\alpha$ , IL-1, or LPS (Delhase et al., 1999). Curiously, the substitution of the same two serines in the IKK $\alpha$  activation loop, whose sequence is almost identical to that of IKK $\beta$ , did not interfere with activation of the IKK holocomplex assembled around the mutant IKK $\alpha^{AA}$  form in response to either TNF $\alpha$  or IL-1 (Cao et al., 2001; Delhase et al., 1999). Similar results were obtained using catalytically inactive (defective in ATP binding) mutants of IKK $\alpha$  and IKK $\beta$ . These findings provided the first clue to the functional heterogeneity of IKK $\alpha$  and IKK $\beta$ . Gene targeting experiments (Li et al., 1999b, 1999d; Takeda et al., 1999; Tanaka et al., 1999) verified that IKK $\beta$ , and not IKK $\alpha$ , is the subunit required for IKK (and NF- $\kappa$ B) activation by proinflammatory stimuli. It is now evident that although it is dispensable for IKK activation in response to proinflammatory stimuli, IKK $\alpha$  is essential for IKK activation by a set of signals that do not affect the IKK $\beta$  subunit (Cao et al., 2001). Mammary epithelial cells and B lymphocytes that express the IKK $\alpha^{AA}$  form instead of the wt protein exhibit defective IKK (and NF- $\kappa$ B) activation in response to two members of the TNF cytokine family: RANK (receptor activator of NF- $\kappa$ B)-ligand (RANKL) and B lymphocyte stimulator (Blys/BAFF), respectively (Cao et al., 2001). By contrast, IKK complexes that contain the IKK $\alpha^{AA}$  subunit but still contain wt IKK $\beta$  display normal responses to TNF $\alpha$ , IL-1, and LPS (Cao et al., 2001). Thus, certain

signals activate IKK via IKK $\alpha$ , while the majority of proinflammatory stimuli target IKK through the IKK $\beta$  subunit.

Exactly how any given signaling pathway targets the IKK complex is still a matter of debate. It has been suggested that cell stimulation with TNF $\alpha$  results in recruitment of IKK complexes to the type 1 TNF $\alpha$  receptor (TNFR1) signaling complex (Devin et al., 2000; Zhang et al., 2000). Although the amount of recruited IKK is relatively small, it may be sufficient to activate the remainder of the IKK pool via IKK-mediated transautophosphorylation. Both IKK $\alpha$  and IKK $\beta$  are efficiently autophosphorylated within the activation loop when overexpressed, and this autophosphorylation is required for their activation (Miller and Zandi, 2001; Zandi et al., 1998). Gene-targeting experiments have established the involvement of several components of the TNFR1 signaling complex, the protein kinase RIP1 and two interchangeable TRAF proteins, TRAF2 and TRAF5 (Devin et al., 2000; Kelliher et al., 1998; Tada et al., 2001), in IKK activation. It was proposed that TRAF2 (and presumably TRAF5) recruit the IKK complex to the receptor, whereas RIP1 is responsible for IKK activation (Devin et al., 2000). However, there is disagreement as to how this recruitment is achieved. While Zhang et al. (2000) showed that IKK $\gamma$  recruited the IKK complex to RIP after TNF $\alpha$  stimulation, Devin et al. (2000) suggested that recruitment is mediated via the IKK catalytic subunits. Although IKK $\gamma$  and especially its C terminus seems to be the logical mediator of IKK recruitment, so far no direct interactions between IKK $\gamma$  and any component of signaling complexes assembled at TNF $\alpha$  or IL-1 receptors were detected. It is possible, however, that upon IKK recruitment to the receptor, the C terminus of IKK $\gamma$  is modified and that this modification somehow triggers IKK activation. Surprisingly, the kinase activity of RIP1 is dispensable for IKK activation, suggesting that RIP1 most likely acts through protein-protein interactions either with IKK or a yet-to-be identified IKK kinase. A similar signaling mechanism is probably used by IL-1 and similar to RIP1, the kinase activity of the IL-1 receptor-associated kinase 1 (IRAK1) is not required for IKK activation (Li et al., 1999c).

Within the emerging paradigm of protein kinases that also function as adapters, another protein kinase involved in IKK activation is PKR, which is required for the response to dsRNA and viral infection (Chu et al., 1999). Interestingly, catalytically inactive PKR is as capable of activating IKK as the wild-type form (Bonnet et al., 2000; Chu et al., 1999). It was proposed that PKR activates IKK through direct protein-protein interaction, possibly via the IKK $\gamma$  subunit (Bonnet et al., 2000). Interestingly, PKR is also required for NF- $\kappa$ B activation by LPS from gram-negative (GN) bacteria (Goh et al., 2000). Instead of IRAK, the GN LPS signal that is generated via Toll-like receptor (TLR) 4 is transduced through the adaptor protein TIRAP/MAL (Fitzgerald et al., 2001; Horng et al., 2001). In that case, PKR may play a role analogous to the one played by IRAK in the case of IL1R and TLR2/6 signaling. dsRNA also acts via TLR3 (Alexopoulou et al., 2001). TLR3 signaling is dependent on MyD88, as is the response mediated by TLR9, which is responsible for IKK and NF- $\kappa$ B activation by ISS-DNA (Hemmi et al., 2000). Curiously, the response to ISS-DNA also requires the DNA-dependent protein kinase,

DNA-PK (Chu et al., 2000). However, the relationships between TLR9 signaling and DNA-PK activation are not clear, but it is possible that DNA-PK may play an analogous role to PKR.

Besides the kinases described above, recent studies have indicated a surprising role for ubiquitination and the MAP3 kinase TAK1 in the activation of the IKK complex (Deng et al., 2000; Wang et al., 2001). Using an *in vitro* system, it was found that activation of the I $\kappa$ B kinase complex by TRAF6 required two biochemically purified activities, TRIKA1 and TRIKA2. While TRIKA1 consists of the ubiquitination proteins Ubc13 and Uev1A, TRIKA2 consists of TAK1 and two other associated proteins, TAB1 and TAB2. Biochemical analysis suggested that nonclassical ubiquitination of TRAF6 on lysine 63 by Ubc13 and Uev1A led to its activation, and such ubiquitinated TRAF6 could then activate the TAK1 complex. The activated TAK1 subsequently activates MKK6 and IKK, thereby turning on both the JNK and NF- $\kappa$ B pathways. Although these studies are provocative and suggest a potentially novel role for ubiquitination in the NF- $\kappa$ B signaling pathway, all of the evidence presented to date rely on *in vitro* or overexpression analysis, and genetic evidence for such regulatory processes is still lacking.

It is plausible that PKR, IRAK1, and RIP1 activate IKK directly via an induced proximity mechanism in which a dimer or a higher oligomer of the activator contacts each IKK $\alpha$ /IKK $\beta$  dimer, increasing their proximity within the higher order IKK complex, and thereby facilitating mutual transautophosphorylation. In such a case, no IKK kinase is needed. Indeed, none of the putative IKK kinases proposed so far, including NIK (Yin et al., 2001), NAK/T2K/TBK (Bonnard et al., 2000), MEKK1 (Xia et al., 2000; Yujiri et al., 2000), Cot/TPK2 (Dumitru et al., 2000), and even the TAB2 component of the TAK1 signaling complex (S. Akira, personal communication), have withstood the gene disruption test—no effects on activation of IKK or induction of NF- $\kappa$ B DNA binding activity have been detected in mice lacking these kinases. One exception may be MEKK3, which is required for IKK activation in response to TNF in cells; however, the exact mechanism by which it activates IKK remains to be established (Yang et al., 2001). Thus, until a role of IKK kinases is firmly established, the induced proximity model remains an attractive and logical possibility.

Another protein kinase whose role in IKK or NF- $\kappa$ B activation has been genetically confirmed is PKC $\theta$  (Sun et al., 2000). Although PKC $\theta^{-/-}$  mice are viable and apparently normal, their peripheral T cells cannot be activated in response to antigen due to a striking deficiency in NF- $\kappa$ B activation (Sun et al., 2000). PKC $\theta$ , however, is not a direct IKK activator, and it is possible that its activity requires two other proteins, Bcl10 and MALT1 (Gaide et al., 2001; Lucas et al., 2001). Intriguingly, the genes encoding both of these proteins are affected by two different chromosomal translocations involved in the same type of mucosal-associated lymphoid tissue (MALT) lymphoma. Such rearrangements result in overexpression of Bcl10 or MALT1 fusion proteins whose overexpression can lead to NF- $\kappa$ B activation (Gaide et al., 2001; Lucas et al., 2001; Uren et al., 2000). Importantly, Bcl10-deficient lymphocytes do not activate NF- $\kappa$ B in response to occupancy of antigen receptors

(Ruland et al., 2001), and coexpression of MALT1 facilitates Bcl10-mediated NF- $\kappa$ B activation (Lucas et al., 2001). MALT1 is a paracaspase that contains a CARD domain known to be involved in aggregation-induced activation. Despite the similarity in phenotypes and function, the exact mechanistic relationships between PKC $\theta$ , Bcl10, MALT1, and IKK remain to be elucidated. Although the substrate for PKC $\theta$  in this signaling cascade needs to be identified, it should be noted that unlike many of the protein kinases discussed above, catalytically inactive PKC $\theta$  is incapable of activating NF- $\kappa$ B (Khoshnan et al., 2000).

An important issue that is yet to be resolved is the exact role of IKK $\gamma$ . Undoubtedly, IKK $\gamma$  is essential to IKK activation. It is also clear that in addition to its role in IKK holoenzyme assembly, IKK $\gamma$  plays a critical signaling role. An attractive hypothesis, mentioned above, is that IKK $\gamma$  directly interacts with distinct IKK activators. Thus, several groups have undertaken the task of identifying IKK $\gamma$ -interacting proteins, and in addition to IKK $\alpha$  and IKK $\beta$ , this approach has resulted in the identification of a small number of potential IKK $\gamma$  binding proteins (Hong et al., 2001; Leonardi et al., 2000). Unfortunately, however, the physiological function of any of these proteins is not yet known. Another possibility that needs to be examined is whether IKK $\gamma$  itself serves as a target for regulated phosphorylation (Carter et al., 2001). Although TNF $\alpha$  stimulation results in increased IKK $\gamma$  phosphorylation, the location of the phosphorylation sites and their function remains to be determined. Even in the case of IKK $\beta$ , where the role of phosphorylation has been determined, some of the observed phosphorylation events have an activating role and some have an inhibitory role (Delhase et al., 1999).

#### Why Does the IKK Complex Contain Two Catalytic Kinase Subunits?

The extensive structural similarity between the IKK $\alpha$  and IKK $\beta$  catalytic subunits, their similar abilities to phosphorylate I $\kappa$ B proteins and their heterodimerization suggested that their functions are likely to be redundant and overlapping. However, the site-directed mutagenesis experiments discussed above suggest that IKK $\alpha$  and IKK $\beta$  may have distinct regulatory functions. This possibility has been confirmed through the generation and analysis of mutant mice that either lack specific IKK subunits or express them as inactive forms.

Disruption of the *Ikk* $\beta$  locus results in midembryonic lethality due to extensive liver apoptosis (Li et al., 1999b, 1999d; Tanaka et al., 1999), a phenotype very similar to that resulting from ablation of the *RelA* gene (Beg et al., 1995). Most importantly, the lethality associated with the loss of either IKK $\beta$  or p65/RelA is prevented by ablation of TNFR1 (Alcamo et al., 2001; Li et al., 1999b, 1999d; Senftleben et al., 2001b), indicating that it is TNF $\alpha$  mediated. These results provided a genetic proof that IKK $\beta$  is required for activation of p65/RelA-containing NF- $\kappa$ B dimers in liver cells and that these complexes are in turn required for expression of antiapoptotic genes whose products suppress TNF $\alpha$ -induced apoptosis. Congruently, IKK $\beta$ -deficient mouse fibroblasts display an NF- $\kappa$ B activation defect not only in response to TNF $\alpha$ , but also in response to IL-1, dsRNA, and ISS-DNA (Chu et al.,



1999, 2000; Li et al., 1999b, 1999d; Tanaka et al., 1999). Furthermore, IKK $\beta$ -deficient thymocytes exhibit defective NF- $\kappa$ B activation and highly attenuated proliferative response following ligation of the T cell receptor (Senftleben et al., 2001b).

Despite the absence of embryonic lethality and their normal development, *Ikk $\beta$ <sup>-/-</sup>Tnfr1<sup>-/-</sup>* double mutant mice fail to thrive and die within 2–3 weeks after birth, suffering from severe opportunistic infections (Senftleben et al., 2001b). A similar immunodeficient phenotype is exhibited by *RelA<sup>-/-</sup>Tnfr1<sup>-/-</sup>* double mutant mice, although these mice survive for up to 2 months, due to a higher level of residual NF- $\kappa$ B activity (Alcamo et al., 2001). *Tnfr1<sup>-/-</sup>* mice are not susceptible to such infections, indicating that the severe immunodeficiency is strictly caused by reduced NF- $\kappa$ B activity. Detailed analysis of *RelA<sup>-/-</sup>Tnfr1<sup>-/-</sup>* mice revealed a defect in neutrophil migration due to insufficient production of chemokines and adhesion molecules by epithelial cells (Alcamo et al., 2001). Adoptive transfer experiments demonstrated that *RelA<sup>-/-</sup>Tnfr1<sup>-/-</sup>* neutrophils exhibit normal migration, and reconstitution of *RelA<sup>-/-</sup>Tnfr1<sup>-/-</sup>* mice with normal myeloid and lymphoid cells did not alleviate their susceptibility to infections (Alcamo et al., 2001). Similar results were obtained in adoptive transfer experiments using either *Ikk $\beta$ <sup>-/-</sup>* or *Ikk $\beta$ <sup>-/-</sup>Tnfr1<sup>-/-</sup>* hematopoietic stem cells (Senftleben et al., 2001b). Collectively these experiments demonstrate that the IKK $\beta$  and p65/RelA-containing NF- $\kappa$ B dimers are required for activation of innate immune responses. Interestingly, the most important innate immune functions of IKK $\beta$  and NF- $\kappa$ B are most likely exerted within the nonhematopoietic compartment, probably in the epithelial cells of tissues, such as lung, liver, and intestinal mucosa, which come in contact with a variety of pathogens. Although the genes that mediate innate immunity remain to be identified, a likely possibility is that NF- $\kappa$ B activation within epithelial cells is required for expression of antimicrobial peptides, such as the  $\beta$  defensins, that provide the first line of defense against microbial infections. In addition, NF- $\kappa$ B is required for the synthesis of chemokines and adhesion molecules, which are needed for recruitment of professional bacteriocidal cells, such as macrophages and neutrophils.

In addition to their undisputable function in activation of innate immune responses, IKK $\beta$  and p65/RelA are required in lymphoid cells for suppression of apoptosis (Grossmann et al., 2000; Horwitz et al., 1997; Senftleben et al., 2001b). Lethally irradiated mice reconstituted with either *Ikk $\beta$ <sup>-/-</sup>* or *Nfkb1<sup>-/-</sup>/RelA<sup>-/-</sup>* hematopoietic stem cells display almost complete absence of B and T lymphocytes at various stages of development and a marked increase in the number of monocytes and granulocytes (Horwitz et al., 1997; Senftleben et al., 2001b). However, analysis of *Ikk $\beta$ <sup>-/-</sup>Tnfr1<sup>-/-</sup>* or *RelA<sup>-/-</sup>Tnfr1<sup>-/-</sup>* newborn mice revealed either a partial decrease in the number of lymphocytes or no decrease at all (Alcamo et al., 2001; Senftleben et al., 2001b). Although NF- $\kappa$ B is not required for lymphocyte development per se, it is required for suppressing lymphocyte apoptosis during early development (Voll et al., 2000) or in response to TNF $\alpha$  production (Senftleben et al., 2001b). NF- $\kappa$ B may also be required for reducing the rate of spontaneous apoptosis in mature B cells by elevating the expression

of Bcl-2 (Grossmann et al., 2000). Through these protective functions and by virtue of its requirement for NF- $\kappa$ B activation, IKK $\beta$  is likely to be an important mediator of inflammation. Experiments using conditional *Ikk $\beta$*  gene knockouts and specific IKK $\beta$  inhibitors are critically needed for evaluating its role in chronic and acute inflammatory diseases.

The initial analysis of *Ikk $\alpha$*  null mice did not reveal any indication of the role of IKK $\alpha$  in the regulation of NF- $\kappa$ B activity and immune responses. Instead, *Ikk $\alpha$ <sup>-/-</sup>* mice exhibited severe morphological abnormalities mostly attributable to defective epidermal differentiation (Hu et al., 2001; Li et al., 1999a; Takeda et al., 1999). However, cells derived from IKK $\alpha$ -deficient animals display normal IKK activation in response to proinflammatory stimuli and relatively normal induction of NF- $\kappa$ B DNA binding activity (Chu et al., 1999; Hu et al., 1999, 2001). Although IKK $\alpha$  performs an essential function in regulating keratinocyte differentiation, this does not depend on its protein kinase activity or its effect on NF- $\kappa$ B (Hu et al., 2001). Due to the early lethality associated with the complete loss of IKK $\alpha$ , adoptive transfer experiments were required to reveal its important and unique function in the lymphoid system, which depends on its protein kinase activity (Kaisho et al., 2001; Senftleben et al., 2001a). Lethally irradiated mice reconstituted with *Ikk $\alpha$ <sup>-/-</sup>* hematopoietic stem cells demonstrate a marked reduction in B cell maturation, antibody production, and germinal center (GC) formation in response to T cell-dependent antigens (Kaisho et al., 2001; Senftleben et al., 2001a). The *Ikk $\alpha$ <sup>-/-</sup>* chimeras also display defective splenic microarchitecture, absence of follicular dendritic cells (FDC), and marginal zone macrophages (Kaisho et al., 2001), as well as absence of Peyer's patches (Senftleben et al., 2001a). As yet, no overt defects in T cell development or proliferation have been reported for these mice (Kaisho et al., 2001; Senftleben et al., 2001a). Thus, IKK $\alpha$  seems to be specifically required for B cell-mediated responses and organization of lymphoid organs.

Does IKK $\alpha$  exert its B cell-specific function through regulation of NF- $\kappa$ B? Interestingly, somewhat similar defects in B cell-mediated responses, GC formation and splenic microarchitecture, were detected in *Nfkb2<sup>-/-</sup>* mice (Caamano et al., 1998; Franzoso et al., 1998). *Nfkb1<sup>-/-</sup>* mice also show defective B cell-mediated responses but normal B cell maturation (Sha et al., 1995). Similar defects but normal B cell development and maturation are also exhibited by *c-Rel<sup>-/-</sup>* mice (Kontgen et al., 1995). Thus, it seems that the specific defect in B cell maturation and formation or organization of lymphoid organs caused by the absence of IKK $\alpha$  is unlikely to reflect a general NF- $\kappa$ B deficiency. Such a conclusion is consistent with recent demonstration of defective induction of NF- $\kappa$ B DNA binding activity in response to LPS or anti-CD40 in *Ikk $\alpha$ <sup>-/-</sup>* B cells (Kaisho et al., 2001). Despite that general defect, the same investigators found relatively normal induction of at least one NF- $\kappa$ B target gene, encoding the antiapoptotic molecule A1 (Kaisho et al., 2001). Indeed, other investigators detected induction of NF- $\kappa$ B DNA binding activity in *Ikk $\alpha$ <sup>-/-</sup>* B cells (Senftleben et al., 2001a). In addition, the defective maturation of *Ikk $\alpha$ <sup>-/-</sup>* B cells was shown not to be due to increased sensitivity to apoptosis that is ex-

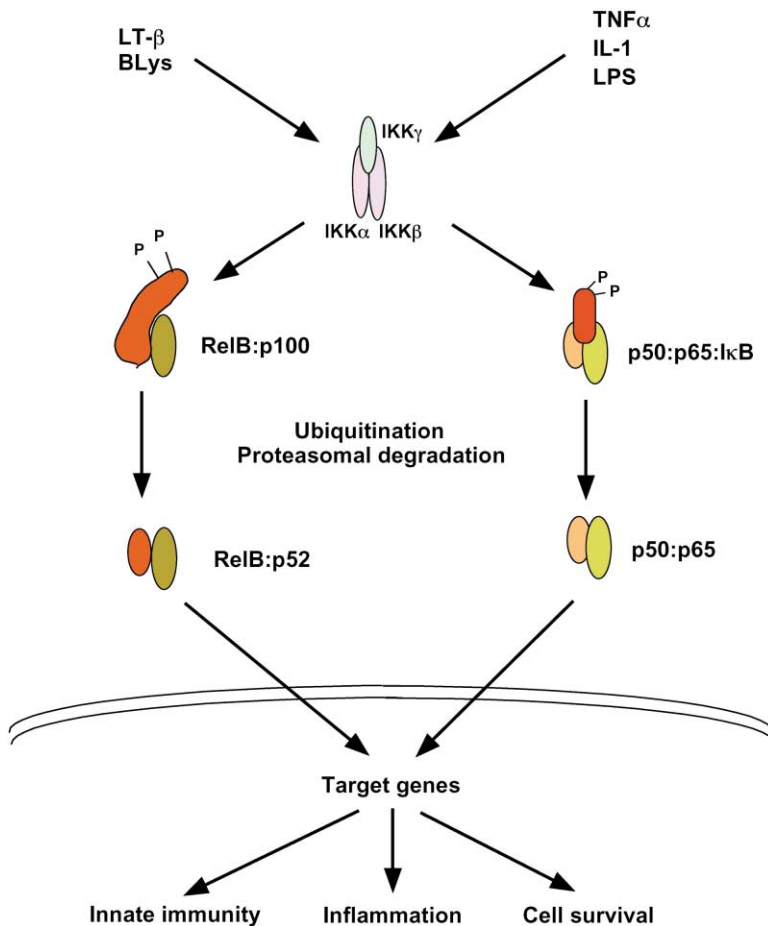


Figure 3. A Model Indicating the Two Signaling Pathways to NF- $\kappa$ B

One pathway is the classical pathway mediated by IKK $\beta$  and leading to phosphorylation of IkB. The other pathway involves IKK $\alpha$  and leads to the phosphorylation and processing of p100, generating p52:RelB heterodimers.

pected in the case of a general NF- $\kappa$ B deficiency, nor can it be suppressed by ectopic Bcl-2 expression (Kaisho et al., 2001).

Most intriguingly, instead of a general NF- $\kappa$ B activation defect, *Ikk $\alpha$ <sup>-/-</sup>* B cells exhibit a specific deficiency in NF- $\kappa$ B2/p100 processing (Figure 3; Senftleben et al., 2001a). Unlike the processing of NF- $\kappa$ B1/p105, which occurs constitutively and is normal in *Ikk $\alpha$ <sup>-/-</sup>* B cells, the processing of NF- $\kappa$ B2/p100 is highly regulated and can be induced in response to ectopic expression of NIK (Xiao et al., 2001). Interestingly, the induction of NF- $\kappa$ B2/p100 processing by ectopic NIK is blocked by the absence of IKK $\alpha$  (Senftleben et al., 2001a), suggesting that IKK $\alpha$  acts downstream of NIK in a pathway that controls NF- $\kappa$ B2/p100 processing. Indeed, knockin mice expressing the IKK<sup>AA</sup> variant, which lacks the activating phosphoacceptor sites that can be phosphorylated by NIK in vitro (Ling et al., 1998), also exhibit defective NF- $\kappa$ B2/p100 processing, defective B cell maturation, and defective lymphoid organ formation (Senftleben et al., 2001a). These results suggest that IKK $\alpha$  may be a specific target for NIK and that, once activated, it may lead to phosphorylation-dependent processing of NF- $\kappa$ B2/p100. Although in vitro studies have demonstrated that IKK $\alpha$  can phosphorylate NF- $\kappa$ B2/p100 on sites that are required for processing, this remains to be shown in vivo. As both *Ikk $\alpha$ <sup>-/-</sup>* and *Ikk $\alpha$ <sup>AA/AA</sup>* B cells contain IKK complexes that can still be activated by LPS,

it is unlikely that the classical IKK complex is involved in NF- $\kappa$ B2/p100 processing. Indeed, NIK-induced NF- $\kappa$ B2/p100 processing is not affected by the absence of either IKK $\beta$  or IKK $\gamma$  (Senftleben et al., 2001a).

Most likely, the processing of NF- $\kappa$ B2/p100 depends on a different IKK-containing complex that may also contain NIK. It is noteworthy that, in addition to its biochemical identification as a component of the IKK complex (DiDonato et al., 1997), IKK $\alpha$  was also identified as a NIK-interacting protein in a yeast two-hybrid screen (Regnier et al., 1997). Although it remains to be determined whether a complex containing both proteins actually exists, the NIK-IKK $\alpha$  pathway seems to be specifically activated by the LT $\beta$  receptor (LT $\beta$ R), a member of the TNFR family. Preliminary results reveal that agonistic anti-LT $\beta$ R antibodies can induce NF- $\kappa$ B2/p100 processing in an IKK $\alpha$ -dependent manner. Furthermore, the phenotype of LT $\beta$ R-deficient mice (Futterer et al., 1998) is similar to that of *Nik<sup>aly/aly</sup>* (Fagarasan et al., 2000; Shinkura et al., 1999) or *Nik<sup>-/-</sup>* (Yin et al., 2001) mice, all of which show extensive defects in lymphoid organ development and organization that may be due in part to insufficient production of B lymphocyte chemoattractant (BLC) by stromal cells (Fagarasan et al., 2000). Interestingly, both *LT $\alpha$ <sup>-/-</sup>* (Ngo et al., 1999) and *Nfkb2<sup>-/-</sup>* (Poljak et al., 1999) mice fail to express BLC. Thus, we predict that BLC is encoded by a specific p52-dependent gene whose production by stromal cells is required for organi-

zation of lymphoid organs. As  $\text{LT}\beta\text{R}$  is expressed in stromal cells rather than in B lymphocytes, and BLC is a chemokine acting via a G protein-coupled receptor that is unlikely to be a potent IKK activator, it appears that another receptor is likely to account for activation of the putative NIK-IKK $\alpha$  signaling complex and induction of NF- $\kappa$ B2/p100 processing in B cells. One possible candidate for this receptor is BAFF-R, a recently discovered Blys(BAFF) receptor, that is also a member of the TNFR family (Thompson et al., 2001; Yan et al., 2001). Recent studies have demonstrated that mice lacking Blys or BAFF-R exhibit defective B cell maturation and lymphoid organ organization similar to the *Ikk $\alpha$ <sup>-/-</sup>* chimeras (Schiemann et al., 2001; Thompson et al., 2001).

Although not formally proven, indirect evidence suggests that defective NF- $\kappa$ B2/p100 processing is responsible for many of the defects in B cell maturation, humoral immunity, and lymphoid organ development present in *Ikk $\alpha$ <sup>-/-</sup>* chimeras or *Nik<sup>-/-</sup>* mice. First, similar defects are exhibited by *Nf $\kappa$ b2<sup>-/-</sup>* mice (Caamano et al., 1998; Franzoso et al., 1998) that are also defective in BLC production (Poljak et al., 1999). Second, expression of NF- $\kappa$ B2 is particularly high in mature B cells (Senftleben et al., 2001a). Third, mice that produce p52 constitutively due to deletion of the 3' portion of the *Nf $\kappa$ b2* gene coding for the I $\kappa$ B-like domain exhibit B cell hyperplasia and enlargement of spleen and lymph nodes (Ishikawa et al., 1997). Finally, chromosomal rearrangements that detach p52 from the I $\kappa$ B-like domain of p100 have been detected in B cell lymphomas (Zhang et al., 1994). It therefore appears that instead of playing a role in the canonical NF- $\kappa$ B activation pathway initiated by proinflammatory stimuli and involving phosphorylation and degradation of I $\kappa$ B proteins, IKK $\alpha$  functions in a specialized pathway based on phosphorylation-dependent processing of NF- $\kappa$ B2/p100 to p52.

In contrast to its processing, the expression of p100 seems to be regulated by the canonical IKK $\gamma$ -dependent NF- $\kappa$ B activation pathway, as NF- $\kappa$ B2 has been identified as an NF- $\kappa$ B target gene. Given the important role of NF- $\kappa$ B2 in B cell biology, it is likely that the two separate IKK $\alpha$ - and IKK $\beta$ -dependent signaling pathways play a central role in providing a molecular linkage between innate and adaptive immune responses. B cell stimulation by LPS or via the antigen receptor may result in upregulation of p100 expression and activation of certain NF- $\kappa$ B target genes. In the absence of p100 processing, this may drive the expansion of antigen-specific but immature B cells. Only within lymphoid organs, such as GCs and Peyer's patches, would B cells be exposed to Blys, which is produced by macrophages. This would lead to their maturation and to the activation of processes such as class switching and affinity maturation.

IKK $\alpha$  is also required for induction of I $\kappa$ B degradation in response to certain stimuli, although it is not essential for the response to common proinflammatory stimuli. A recent study demonstrated that *Ikk $\alpha$ <sup>AA/AA</sup>* mice exhibited defective development of the mammary gland during pregnancy (Cao et al., 2001). This defect was attributed to the inability of *Ikk $\alpha$ <sup>AA/AA</sup>* mammary epithelial cells to respond to RANKL with induction of classical NF- $\kappa$ B DNA binding activity (p50:p65 heterodimers). Correspondingly, *Ikk $\alpha$ <sup>AA/AA</sup>* mammary epithelial cells exhibit

defective expression of the NF- $\kappa$ B-dependent gene cyclin D1, which is required for proliferation of the mammary epithelium during pregnancy (Cao et al., 2001). In this case, IKK $\alpha$  may function as a component of the classical IKK complex that is specifically required for its activation by RANKL but not TNF $\alpha$ , IL-1, or LPS (Cao et al., 2001). It remains to be determined, however, whether IKK $\alpha$  is required for other RANKL-regulated processes, such as osteoclast and T cell differentiation (Kong et al., 1999).

Unlike the situation in B cell development discussed above, it is not entirely clear what biological advantage the functional separation of IKK $\alpha$ - and IKK $\beta$ -dependent NF- $\kappa$ B signaling in mammary epithelial cells would provide. As the mammary gland is a recent adaptation unique to mammals, it is possible that this separation is simply due to recruitment of RANK signaling to the control of mammary gland development. Thus, the real cause for functional separation between IKK $\alpha$  and IKK $\beta$  may lie within another cell type whose destiny depends on RANK signaling—the macrophage. It would be worthwhile examining whether macrophage proliferation and activation are dependent on IKK $\beta$ , whereas macrophage differentiation into more mature cell types depends on RANK at IKK $\alpha$ .

## Conclusions

Of the many unanswered questions concerning various aspects of NF- $\kappa$ B regulation and function, we have highlighted here only a few areas for discussion. Other important questions that we were unable to cover include understanding the exact mechanism of p105 and p100 processing, defining the mechanism underlying transient versus persistent NF- $\kappa$ B activation, identifying the mechanisms involved in the termination of NF- $\kappa$ B activation, determining the role of modulators such as the  $\kappa$ B-Ras proteins, and elucidating the mechanisms underlying constitutive activation of NF- $\kappa$ B in different tumors. However, the issues that we have highlighted, in our opinion, represent areas of current, active research, and findings in these areas will contribute significantly to our further understanding of NF- $\kappa$ B biology.

We also have not discussed how different NF- $\kappa$ B dimers activate distinct sets of NF- $\kappa$ B target genes, something that is suggested by the distinct phenotypes of knockout mice lacking distinct NF- $\kappa$ B subunits. It is also not clear which of the target genes activated by p65/RelA are most critical for the suppression of apoptosis. As recently discussed, the exact mode by which NF- $\kappa$ B inhibits apoptosis may vary from one cell type to another (Karin and Lin, 2002). Despite the many remaining uncertainties and questions, it has become apparent that the IKK complex plays a central role in regulation of NF- $\kappa$ B activity and function. It is also clear that the unusual composition of IKK, with two distinct catalytic subunits and one regulatory subunit, is intimately related to its multifunctional role. The classical IKK complex, composed of IKK $\alpha$ , IKK $\beta$ , and IKK $\gamma$ , is required for nuclear translocation of NF- $\kappa$ B heterodimers in response to a diverse collection of stimuli. While responses to proinflammatory stimuli, such as TNF $\alpha$ , IL-1, LPS, and dsRNA, are largely dependent on the IKK $\beta$  catalytic subunit, responses to other stimuli, such as RANKL and possibly

Blys/BAFF or LT $\alpha/\beta$ , depend on the IKK $\alpha$  catalytic subunit. Whereas IKK $\beta$  activation leads to nuclear translocation of NF- $\kappa$ B heterodimers by inducing I $\kappa$ B phosphorylation and degradation, the IKK $\alpha$  subunit is involved in formation of another signaling module together with NIK that is required for processing of NF- $\kappa$ B1/p100. This module is specifically activated by LT $\alpha/\beta$  through the LT $\beta$ R and by Blys/BAFF through BAFF-R. As NF- $\kappa$ B2/p100 has an I $\kappa$ B-like function and the basal level of processed p52 in nonstimulated cells is very low, it is likely that inducible NF- $\kappa$ B2/p100 processing results in the nuclear translocation of specific p52-containing NF- $\kappa$ B heterodimers, such as p52:RelB. This processing-based pathway is likely to be responsible for activation of a specific subset of NF- $\kappa$ B target genes. This pathway is not involved in innate immunity or inflammation but instead plays a central role in mediating cell-cell interactions that are critical for lymphoid organ development.

As is clear from this review, there is a lot that remains to be understood about regulation of NF- $\kappa$ B. However, we believe that the convergence of genetic and biochemical approaches that are being currently utilized will provide us with answers in the near future that will shed light on many of the remaining mysteries of the mammalian NF- $\kappa$ B system.

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