

CHAPTER 2

NF- κ B Signal Transduction by IKK Complexes

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Abstract

Transcription factor NF- κ B plays a major role in many physiological and pathological processes while its regulation is best understood in inflammatory and immune system. The central event in NF- κ B signaling pathway is the activation of IKK complex, the convergent point of diverse NF- κ B activation signaling. This review addresses the cell signaling of IKK and NF- κ B activation in response to various immune and inflammatory stimuli as revealed by the analysis of mice and cells lacking specific signaling transducers.

NF- κ B, I κ B, IKK and the Canonical Pathway for NF- κ B Activation

NF- κ B is a master transcription factor that plays a major role in inflammatory and immune response.¹ It was originally found in nuclei of B cells and named for its ability binding the κ -chain enhancer of immunoglobulin in B cells. NF- κ B was later found in the cytoplasm of all cell types, where it enters the nucleus upon stimulation. NF- κ B transcription factors are evolutionarily conserved from insects to mammals. In mammals, the NF- κ B family consists of five members (p65/RelA, RelB, c-Rel, p50/NF- κ B1 and p52/NF- κ B2). These proteins share an N-terminal domain of about 300 amino acids, which bears homology to the product of the *v-rel* oncogene, the Rel homology domain (RHD), and includes regions for DNA binding, dimerization and nuclear translocation (Fig. 1). DNA binding by NF- κ B requires dimerization and most members of this family form both homo- and heterodimers except for RelB, which forms only heterodimers with p50 or p52. Mammalian NF- κ B proteins can be classified into two groups; the first group, consisting of p65 (RelA), RelB and c-Rel, are expressed as mature proteins and possess a transcriptional activation domain at their C-termini. NF- κ B dimers containing any one of these subunits can activate target gene transcription upon induction by certain stimuli. The second group consists of p50 (NF- κ B1) and p52 (NF- κ B2), which are first expressed as large precursors p105 and p100, respectively. NF- κ B1 precursor p105 is constitutively processed to produce p50, whereas p52 is proteolytically released from p100 only upon stimulation. Both p50 and p52 lack a potent transcriptional activation domain and therefore cannot activate transcription as homodimers, or as p50/p52 heterodimers. In fact, p50/p52 dimers may suppress expression of NF- κ B target genes.

The C-termini of p105 and p100 contain multiple ankyrin repeats, which are required for association with NF- κ B and are the distinguishing structural feature of the I κ Bs, the specific inhibitors of NF- κ B (Fig. 1). Therefore, p105 and p100 can serve an I κ B-like function by

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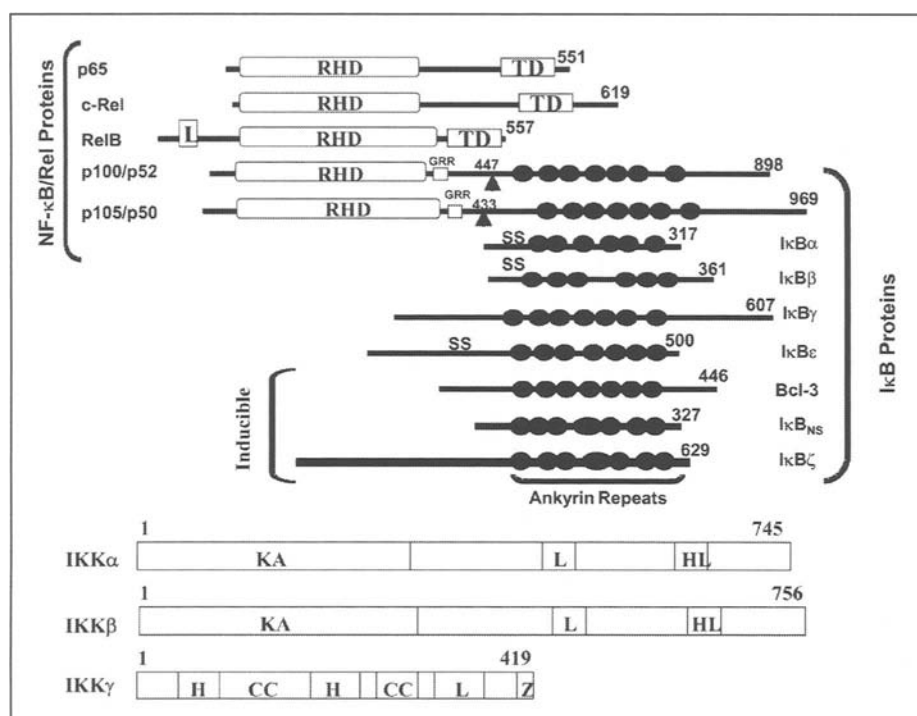


Figure 1. Mammalian NF- κ B, I κ B and IKK proteins. CC: coiled coil; GRR: glycine rich repeat; H: α -helix; HLH: helix-loop-helix; L: leucine zipper; RHD: Rel homology domain; SS: serine phosphorylation sites; TD: transactivation domain; Z: zinc finger. Modified from Li ZW, Rickert RC and Karin M. Genetic dissection of antigen receptor induced-NF-kappaB activation. *Mol Immunol* 2004; 41(6-7):701-714.

retaining RelA, RelB or c-Rel in the cytoplasm. Three major mammalian I κ B proteins, I κ B α , I κ B β and I κ B ϵ , have been identified.¹ These I κ Bs have overlapping yet distinct inhibitory specificity and thus can differentially inhibit NF- κ B dimers. In addition, the C-terminal portion of p105 can be expressed as an independent transcript that encodes I κ B γ , which is expressed only in the lymphoid cells. Another mammalian I κ B family member is the nuclear protein Bcl-3.¹ Although it contains ankyrin repeats, Bcl-3 functions as a transcriptional activator with p50 or p52 homodimers, rather than an inhibitor of NF- κ B. This activity may be caused by Bcl-3-mediated displacement of p50 or p52 homodimers from NF- κ B binding site to allow binding of NF- κ B molecules with transactivation domains, such as p65, c-Rel and RelB. Alternatively, Bcl-3 may also activate gene transcription by its own transactivation domain.² Bcl-3 production is inducible and is required for humoral immune response. Other two inducible I κ B family members are I κ B ζ (also called MAIL or INAP) and I κ B_{NS}.³⁻⁶ I κ B ζ is required for Toll-like receptor (TLR) and interleukin 1 (IL-1) receptor (IL-1R) activation induced production of IL-6,⁷ and I κ B_{NS} is induced by TCR (T cell receptor) activation,⁶ suggesting that other inducible I κ Bs may exist and respond to diverse stimulation. However, how these inducible I κ Bs function has yet to be determined.

In addition to the ankyrin repeats, the C-terminal acidic region of I κ Bs is necessary for their inhibitory activity. The PEST motif in the C-terminal acidic region of I κ B is the target site of I κ B phosphorylation that is responsible for the basal turnover of these proteins and their induced degradation in response to UV irradiation.⁸ The I κ Bs inhibit NF- κ B activity by masking the nuclear localization signal (NLS) of NF- κ B, thereby retaining NF- κ B in the cytoplasm

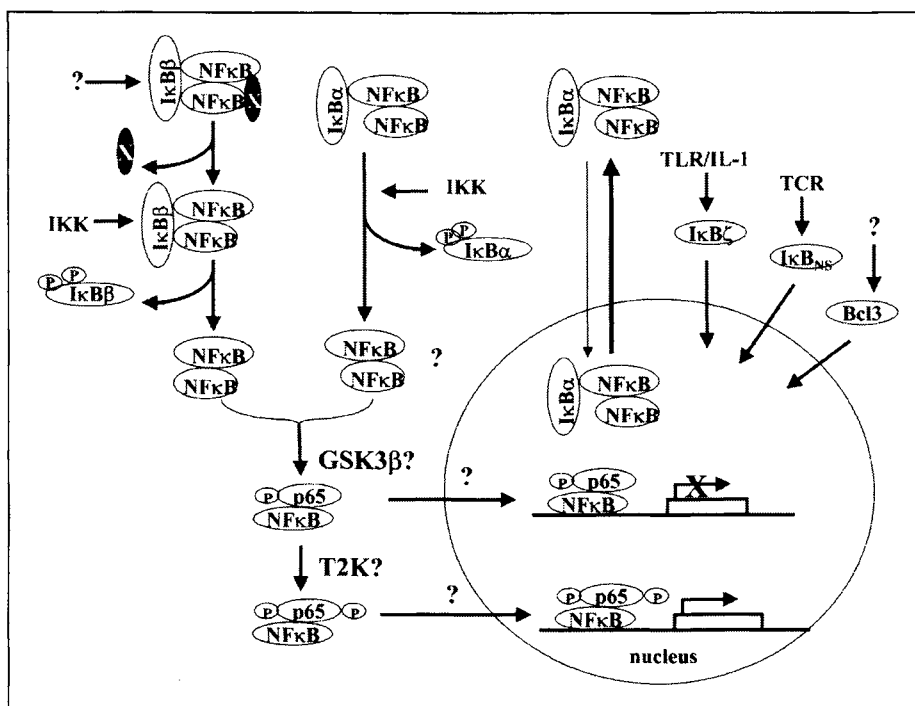


Figure 2. Regulation of NF- κ B activity by I κ Bs and protein kinases. I κ B α :NF κ B complex shuttles between the cytoplasm and nucleus although majority of the complex is in the cytoplasm. The I κ B β :NF- κ B complex is retained in cytoplasm by I κ B β and protein X such as κ B-Ras. Upon stimulation, unidentified molecule removes X from I κ B β :NF- κ B complex. IKK phosphorylation of I κ Bs leads to the ubiquitination and degradation. Released NF- κ B is phosphorylated and then induces gene expression. Nuclear import of inducible I κ Bs (Bcl3, I κ B ζ , I κ B_{NS}) occurs only upon certain stimuli. For p65 containing NF- κ B, GSK3 β phosphorylation might enhance DNA binding, and further T2K phosphorylation might lead to gene transcription. Question markers indicate unidentified or unconfirmed molecule(s). Modified from Li ZW, Rickert RC and Karin M. Genetic dissection of antigen receptor induced-NF-kappaB activation. *Mol Immunol* 2004; 41(6-7):701-714.

(Fig. 2). There are two variations of this model.⁹ One mechanism is used by I κ B β and causes cytoplasmic retention of NF- κ B due to the masking of two NLSs on NF- κ B dimers. Interaction between the NF- κ B:I κ B β complex and the small guanosine triphosphatases κ B-Ras-1, -2 also contribute to NF- κ B activation. When binding to κ B-Ras, I κ B β cannot be phosphorylated by IKK, thus blocking the NF- κ B activation signal from IKK.¹⁰ I κ B α and I κ B ϵ , which both mask one NLS of NF- κ B, utilize the other mechanism in which the 2nd NLS of NF- κ B and the nuclear export signal (NES) of I κ B α or I κ B ϵ causes the I κ B:NF- κ B complex to shuttle between the nucleus and cytoplasm. Recently, Moorthy et al suggested that I κ Bs, including I κ B γ (the c-terminus of p105), utilize the same binding mechanism, and the localization of I κ B:NF- κ B complex might be controlled by other protein(s).¹¹ It was suggested that κ B-Ras is a protein that interacts only with I κ B β but not other I κ B family members. The interaction of κ B-Ras with the NF- κ B:I κ B β complex causes the binding of two NF- κ B NLS motifs by I κ B β . Removal of κ B-Ras release one NLS and leads to nuclear import of the NF- κ B:I κ B β complex.¹² If this is true, free κ B-Ras should be detected upon NF- κ B activation stimuli. The differential control between I κ B α and I κ B β may lead to biphasic activation of NF- κ B. As a target gene of NF- κ B, I κ B α is promptly upregulated upon NF- κ B activation and therefore

controls the fast transient activation of NF- κ B, whereas I κ B β , whose transcription is not controlled by NF- κ B, controls the persistent activation of NF- κ B.¹³ Two MAP3Ks, MEKK3 and MEKK2 were suggested to regulate the biphasic activation of NF- κ B upon TNF α (tumor necrosis factor alpha) and IL-1 α by participating in assembling of I κ B α :NF- κ B/IKK and I κ B β :NF- κ B/IKK complex, respectively.¹⁴

In response to extracellular stimuli, the I κ Bs are rapidly phosphorylated due to activation of a protein kinase complex called the I κ B kinase (IKK).¹⁵ This phosphorylation event targets the I κ Bs for polyubiquitination and degradation by the 26S proteasome and thereafter the release of NF- κ B dimers that translocate to the nucleus to regulate gene transcription. IKK (Fig. 1) contains two closely related catalytic subunits, IKK α and IKK β , which contain a protein kinase domain at their N-terminal portion, whereas their C-terminal portion contains protein interaction motifs such as a leucine zipper (LZ) and a helix-loop-helix (HLH) domain. IKK α and IKK β can both directly phosphorylate I κ Bs and their activity depends on dimerization through their leucine zipper motifs. In addition, IKK activity also depends on the HLH motif that may act as an intramolecular activator of the kinase domain. While the major native IKK complex is based on IKK α :IKK β heterodimers, *in vitro*, both IKK α and IKK β can also form functional homodimers. IKK β is the major kinase controlling canonical pathway of NF- κ B activation, in which phosphorylation of I κ B by IKK release NF- κ B to enter nuclear and regulate gene expression. The native IKK complex also contains a regulatory subunit, IKK γ that can form homodimers and is necessary for assembly of the IKK complex and recruitment of upstream activators to the IKK complex. The C-terminal domain of IKK γ is essential for IKK kinase activity,¹⁶ while the N-terminus is required for binding of IKK γ to the catalytic subunits and therefore is also important for IKK activity.¹⁷ Recently, it was found that the C-terminal oligomerization domain of IKK γ is required for dimerization whereas tetramerization, which enhances IKK kinase activity, needs the N-terminal domain.¹⁸ Consistent with this finding, Weil et al found that the IKK γ N-terminus is sufficient for IKK and NF- κ B activation when recruited to the plasma membrane.¹⁹ Although reports identifying signaling events that only require IKK α or IKK β are beginning to emerge, the similar phenotypes of IKK β -null and IKK γ -null mice strongly suggest that IKK γ is required for activation of IKK β .²⁰ IKK α ^{-/-}, IKK β ^{-/-} double knockout mice showed the same phenotype as IKK γ knockout mice, suggesting that IKK γ may also control IKK α activation in the context of the tri-subunit IKK complex. IKK γ -deficient mice die earlier than IKK α - or IKK β -deficient mice, making it difficult to precisely determine the function of IKK γ in a variety of physiological processes. Hopefully, the generation of conditional IKK γ knockout mice will circumvent this difficulty. Most recently, another protein, ELKS (for the relative abundance of its constitutive amino acids: glutamic acid (E), leucine (L), lysine (K), and serine (S)), was suggested to be an IKK regulatory subunit.²¹ Knocking down ELKS by RNA interfering leads to defect in NF- κ B activation, including reduction in IKK kinase activity, I κ B phosphorylation and degradation, NF- κ B DNA binding activity, NF- κ B targeting gene expression and the protection of cell death induced by TNF α . It was suggested that ELKS functions probably by recruiting I κ B α to the IKK complex. However, the physiological function of ELKS remains to be explored.

IKK α and Noncanonical Pathway for NF- κ B Activation

In addition to the canonical NF- κ B activation pathway that is mostly dependent upon IKK γ -regulated IKK β activation, I κ B phosphorylation and degradation, and then NF- κ B activation,¹⁵ there are at least two situations in which NF- κ B activation was reported to depend only on IKK α (Fig. 3). One is IKK α -dependent p100 processing, which is believed to be the mechanism for LT β (lymphotoxin beta) and Blys (B lymphocyte stimulator, also called BAFF, TALL-1, zTNF4 or THANK) induced NF- κ B activation.⁹ Ligation of the LT β R (LT β receptor) or Blys receptor (BR3) leads to NIK (NF- κ B-inducing kinase) activation which in turn phosphorylates IKK α and thereby activates NF- κ B by phosphorylating p100 and causing the release of p50:RelB dimers. Indeed, NIK-deficient mice are defective in LT β induced NF- κ B

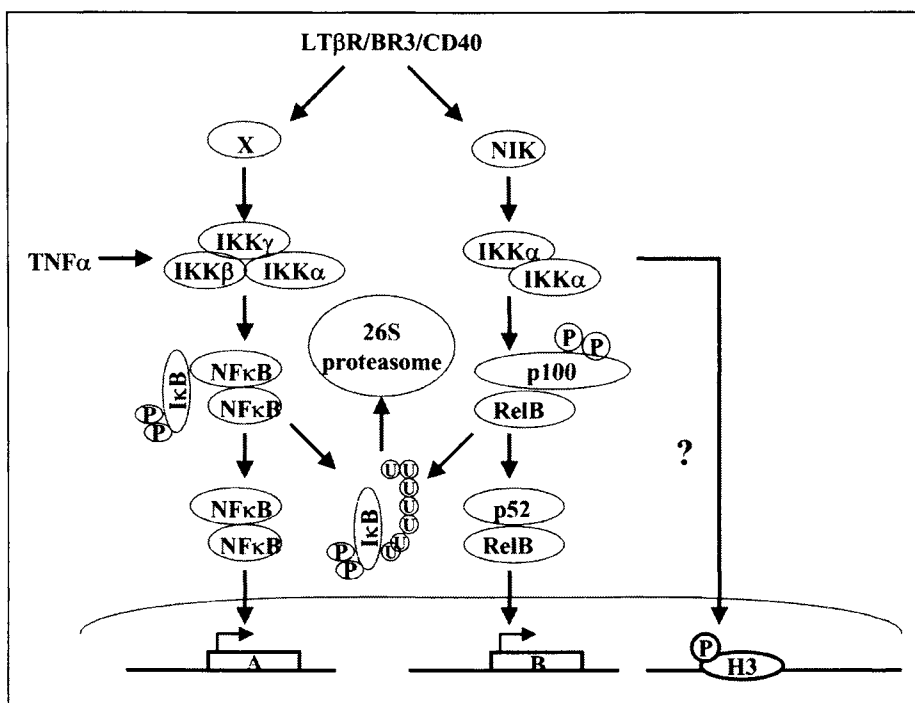


Figure 3. NF- κ B activation is differentially regulated by IKK α and IKK β . Upon LT β /Blys/CD40L stimulation, their receptor LT β R/BR3/CD40 activates NIK and thereafter IKK α homodimers, which in turn phosphorylate p100 and lead to p100 processing, and release p52/RelB heterodimers. An unidentified X signal pathway (it contains TRAF6 for CD40 pathway) activates the IKK holoenzyme composed of all three subunits. In most cases, the activity of this complex depends on IKK β . Modified from Li ZW, Rickert RC and Karin M. Genetic dissection of antigen receptor induced-NF-kappaB activation. *Mol Immunol* 2004; 41(6-7):701-714.

activation.²² IKK α -deficient mice show defective p100 processing in B cells and this might be due to a defect in the phosphorylation of p100 by IKK α .²³ In the case of Blys signaling, *in vivo* data also support this NIK-IKK α -p100 model. Blys knockout mice showed reduced generation of mature follicular B cells.²⁴ This phenotype is also observed in IKK α ^{-/-} fetal liver transplanted mice and in NF- κ B2^{-/-} mice.^{23,25-27} Blys/BR3 signaling promotes p100 processing and thereafter prevents apoptotic B cell death. A BR3-Fc fusion protein, which blocks BR3 signaling, inhibits p100 processing and attenuates the development of autoimmune disease in a mouse model.²⁸ Blys-induced p100 processing and NF- κ B activation are impaired in NIK mutant B cells, and p100 processing is independent of IKK γ , the regulator of the canonical pathway of NF- κ B activation.²⁹ The involvement of IKK α in Blys signaling, however, has not been determined using IKK α -deficient B cells. In NF- κ B2^{-/-} B cells, other NF- κ Bs still respond to Blys stimulation.²⁹ This seems to be not simply due to the compensation of other NF- κ B components since Blys-mediated cell survival is impaired in IKK β -deficient B cells (Z.W. Li, unpublished result). Together, these findings suggested that IKK α is not the only signaling molecule that regulates Blys-mediated B cell survival. With respect to LT β R stimulation, induction of some NF- κ B target genes requires NIK and IKK α , whereas expression of other genes depends only on IKK β and, presumably, IKK γ . RelB upregulation and p100 processing as well as translocation of p52:RelB dimers into the nucleus depend only on NIK and IKK α . However, p100 expression is controlled by RelA and IKK β .³⁰ Consistent with these

findings, LT β R stimulation leads to I κ B α degradation and a shift of DNA binding molecules from RelA- to RelB-containing NF- κ B dimers. Prior activation of the IKK β -dependent pathway results in upregulation of p100:RelB dimers available for IKK α -induced processing.³¹ Saccani et al also found that upon proinflammatory stimulation, NF- κ B mediated gene transcription in monocyte-derived dendritic cells is fine-tuned by exchange of NF- κ B dimers.³² However, the mechanism controlling this dimer exchange is still unclear. CD40L is another NF- κ B activator that utilizes noncanonical pathway,³³ although it also utilizes canonical pathway.³⁴ CD40 was known to recruit TRAF6 (TNF receptor associated factor 6) for NF- κ B activation as justified by analysis of TRAF6 knockout mice.³⁵ It would be interesting to determine how TRAF6 differentially affects both canonical and noncanonical pathways that activate NF- κ B through recruitment of IKK β and IKK α , respectively.

The second mechanism proposed for IKK α -regulated NF- κ B activation is the phosphorylation of histone H3 residue serine 10 by IKK α .^{36,37} Although both groups suggested that IKK α and not IKK β , is the kinase phosphorylating this residue *in vitro*, more work is needed to demonstrate this *in vivo* and resolve a number of questions raised from the discrepancy between the *in vitro* evidence for H3 phosphorylation by IKK α and contrasting *in vivo* findings.³⁸ Most prominently, it is well established that IKK α -deficiency in mice results in postnatal death but normal TNF α signaling, whereas IKK β -deficiency in mice results in liver apoptosis due to defective TNF α -dependent NF- κ B activation.⁹ A crucial question therefore is why the phenotype of IKK α ^{-/-} mice is so different from that of IKK β ^{-/-} mice if IKK α phosphorylation of histone H3 is critical for TNF α induced NF- κ B activation. It is most likely that IKK α -dependent H3 phosphorylation is of little physiological relevance *in vivo*. Recently, it was found that the skeletal morphological defect in IKK α null mice was attributed to failed epidermal differentiation that is regulated by kinase-independent functions of IKK α .³⁹ Although this defect was related to increased FGF8 (fibroblast growth factor 8) expression, how IKK α inhibits FGF8 expression is still a myth.³⁹ Interestingly, nuclear translocation of IKK α is required by this inhibitory function. This finding suggested that IKK α may do play a role by functioning in nucleus.

Modification of NF- κ B and Its Signaling Molecules

Phosphorylation of NF- κ B subunits also contributes to the regulation of NF- κ B activation by facilitate the recruitment of various transcription cofactors.^{9,40} Several kinases, including PKAc (catalytic subunit of protein kinase A), MSK1 (mitogen- and stress-activated kinase-1), RSK1 (ribosomal subunit kinase-1), PI3K/AKT, CKII (casein kinase II), GSK3 β (glucose synthase kinase 3 β), T2K (TRAF2-associated kinase, also called TBK or NAK), IKK, PKC ζ (protein kinase C) and NIK, were suggested to phosphorylate NF- κ B subunits,^{9,40} and GSK3 β , T2K, IKK, PKC ζ and NIK were confirmed by gene targeting to be essential for NF- κ B-regulated gene transcription by particular stimuli.²⁰ While additional evidence suggests that IKK, PKC ζ and NIK act upstream of I κ B, GSK3 β and T2K are the more likely candidates to act downstream of IKK and phosphorylate p65.⁹ In response to a variety of stimuli, cells deficient in either GSK3 β or T2K exhibit normal I κ B degradation and NF- κ B nuclear translocation, but are defective in NF- κ B target gene transcription. Furthermore, either GSK3 β or T2K knockout mice die during mid-gestation due to massive liver apoptosis,^{41,42} which is the same phenotype observed in IKK β ^{-/-} or p65^{-/-} mice.⁴³⁻⁴⁶ However, NF- κ B DNA binding activity is only affected in the GSK3 β knockout, and not in the T2K knockout (Fig. 2), suggesting that these kinases differentially control p65 activity downstream of I κ B degradation. To provide further evidence for the phosphorylation of p65 by GSK3 β or T2K, it will be important to compare p65 phosphorylation in wild type and GSK3 β - or T2K-deficient cells in response to particular stimuli. Further *in vivo* data to support this hypothesis could be generated by attempting to rescue the GSK3 β - or T2K-deficiencies by overexpression of constitutively activated p65.

Acetylation of RelA is also reported to affect NF- κ B activation, perhaps by regulating the interaction between newly synthesized I κ B and RelA. This acetylation is reversible and

ligase in NF- κ B activation has yet to be explored. Another NF- κ B signaling regulator A20 was reported to be a de-ubiquitination/polyubiquitination enzyme downregulating NF- κ B signaling by de-ubiquitinating RIP (receptor interacting protein) and target it for degradation by polyubiquitination.⁵³ The most interesting topic might be the ubiquitination of IKK γ . Upon nuclear export inhibitor Leptomycin B treatment, it was found that IKK γ competes with p65 and IKK α for binding to the N terminus of CBP and therefore leads to transcriptional repression of the NF- κ B pathway.⁵⁴ Whether this is a truly physiological situation remains to be clarified since IKK γ -deficient mice show the phenotype similar to IKK β - or p65-deficient mice, although it is possible that IKK γ has other function in addition to the activation of IKK β and p65 upon TNF α stimulation during liver development. The existence of IKK-unbound free IKK γ has been reported by others.⁵⁵ These free IKK γ molecules shuttle between cytoplasm and nucleus. Upon genotoxic stress, free IKK γ is sumoylated in an ATM (ataxia telangiectasia mutant) independent manner. The sumoylation leads to nuclear localization of IKK γ . Although IKK γ is later desumoylated, this modification is required by the sequential ubiquitylation in an ATM-dependent manner and ultimately activation of IKK in the cytoplasm as evidenced by the ATM RNA interfering knocking down analysis. This work proposed a novel mechanism for NF- κ B activation upon genotoxic stress. The reality of this novel mechanism remains to be testified by analyzing the sumoylation modification enzymes.

Inhibition of the family cylindromatosis tumor suppressor gene (CYLD) enhances the activation of NF- κ B. CYLD binds to IKK γ and regulate IKK activity by de-ubiquitination of TRAF2, and to a less extent, of TRAF6.⁵⁶⁻⁵⁸ CYLD may function through binding to TRIP (TRAF-interacting protein) and stabilize TRIP by removing the ubiquitins and thereby blocking NF- κ B activation (Fig. 4).⁵⁹ The ubiquitination of IKK γ was also suggested to be regulated by c-IAP1 in TNF α activation of NF- κ B.⁶⁰ Using purified protein and a cell-free system, it was found that Bcl-10 activates NF- κ B through the intrinsic ubiquitin ligase activity of paracaspase MALT1 (mucosa associated lymphoid tissue),⁶¹ or through MALT1 to induce oligomerization and activation of TRAF6 (Fig. 4).⁶² TAK1 (transforming growth factor β -activated kinase 1) is further required to phosphorylate IKK β in response to TCR activation.⁶² Although different groups proposed unidentical model regarding IKK γ ubiquitination in the same Bcl-10 signaling pathway, the above work established signal flow chart from Bcl-10, MALT1, TRAF6, TAK1 and IKK to NF- κ B activation, and emphasized the significance of IKK γ ubiquitination. Since the essential role of TRAF6 in TCR activation of NF- κ B does not match the phenotype of TRAF6 knockout T cells, any significant functional defect of that has not been reported, there might be other missing piece(s) in this NF- κ B activation signaling pathway, or compensation from other molecule such as TRAF2 as suggested.⁶² Analysis of NF- κ B activation in TRAF2 $^{-/-}$, TRAF6 $^{-/-}$ double knockout T cells would be very helpful to elucidate this signaling pathway. It is also interesting to see how TRAF2 and TRAF6 function in TCR activation of NF- κ B without affecting TNF α and IL-1 signaling wherein these two molecules were required for NF- κ B activation.

Molecules Involved in Multiple NF- κ B Activation Signaling Pathway

NF- κ B activation signaling pathway is best understood in the immune and inflammatory system. Genetics dissection of antigen receptor induced-NF- κ B activation was recently reviewed.⁶³ Receptors and adaptors for NF- κ B were also reviewed in this book (see Chapter 3). Recent studies suggested that certain signaling molecules could be utilized by different pathways to activate IKK (Fig. 4). Following receptor-proximal signaling events upon TCR or BCR activation, different PKC isoforms are utilized to induce NF- κ B activation during T cell and B cell development. In pro-B cells, preBCR activation of NF- κ B is regulated by PKC λ through both IKK α and IKK β .⁶⁴ In mature B cells, BCR activation of NF- κ B may be regulated by PKC β through IKK α .^{65,66} It is important to note, however, that an additional IKK α -mediated mechanism other than increased NF- κ B DNA binding activity must account for the PKC β -associated defect since DNA binding by NF- κ B is not the major consequence of PKC β

inactivation.⁶⁴ This is also different from PKC θ -regulated NF- κ B activation in T cells, which utilizes IKK β instead of IKK α .⁶⁷

Downstream signaling from PKC to NF- κ B activation is believed to be mediated by CARMA1 (CARD carrying member of the MAGUK family proteins 1), Bcl10 and MALT1,⁶⁸ although how PKC activates CARMA1 and how MALT1 activates IKK remain to be determined *in vivo*. Of notice, TNF α - and IL-1- mediated NF- κ B activation is not affected by CARMA1-, Bcl10- or MALT1-knockout, suggesting that these three signaling molecules are unique for a PKC-dependent pathway. Interestingly, although all of these three molecules are required for NF- κ B activation induced by antigen receptor ligation, their functions in lymphocyte development are different. Only B cell and not T cell development is defective in mice deficient in CARMA1.⁶⁹ However, both B and T cell development are defective in Bcl10 and MALT1 knockout mice.⁷⁰⁻⁷² These findings suggest that in addition to CARMA1, other molecules might be involved downstream of PKC in T cells, which may also recruit Bcl10 and MALT1 to induce NF- κ B activation. Ruland et al also suggested that except for MALT1, other molecule might exist in B cells to relay BCR activation signal to NF- κ B activation since the B cell development is only affected moderately in MALT1 deficient mice.⁷²

Recent progress suggested that some known NF- κ B signaling transducers might be involved in several signaling pathway, although further *in vivo* studies are needed to verify it. The essential role of RIP for NF- κ B activation has been established long time ago. RIP1, the NF- κ B activator in TNF α signaling pathway,⁷³ was recently reported to be essential for TLR3-mediated NF- κ B activation.⁷⁴ It would be interesting to determine how the RIP1 downstream signaling molecules function in TLR3 signaling without affecting TNF α signaling pathway. It was also reported that RIP1 is essential for DNA-damage-induced NF- κ B activation by inducing I κ B α degradation, suggesting that this activation may go through IKK.⁷⁵ Indeed, upon DNA damage, it was confirmed that RIP forms a complex with IKK, and I κ B α degradation requires IKK β . Interestingly, the kinase activity of RIP is not required and IKK activation was not confirmed by kinase assay. Although RIP is involved in both TNF α and DNA damage-induced NF- κ B activation, other signaling molecules, such as TNFR1 (TNF receptor 1), TRAF2, TRAF5 and FADD (Fas-associated death domain protein) are involved only in TNF α induced NF- κ B activation. Although ATM was found to be required for the formation of RIP-IKK complex upon DNA damage, how does RIP activate IKK remains to be solved.⁷⁵

RIP2, another RIP family protein, is involved in TLR-mediated NF- κ B activation.^{76,77} RIP2^{-/-} cells and mice exhibit impaired responses, including defective NF- κ B activation, cytokine production and are resistant to endotoxic shock in response to LPS, dsRNA and peptidoglycan stimulation. It was suggested that RIP2 plays a role in signaling by TLR2/4/9 and Nod1.^{76,77} RIP2 is also involved in TCR signaling to NF- κ B activation. RIP2-deficient T cells show severely reduced NF- κ B activation upon TCR engagement, as well as some other defect in TCR signaling, T cell differentiation and function,^{76,77} whereas RIP1 is important for TNFR2 signaling in thymocyte development and apoptosis, but is not required for thymocyte proliferation.⁷⁸ How RIP1 and RIP2 are involved in TCR signaling differentially is not yet clear. Ruefli-Brasse et al reported that upon TCR activation, RIP2 associates and phosphorylates Bcl-10 and therefore involved in TCR mediated NF- κ B activation.⁷⁹

Different from other RIP family members, RIP3 is dispensable for NF- κ B activation by several NF- κ B activators, including the engagement of TCR, BCR, TNFR1, TLR2 and TLR4.⁸⁰ In contrast, RIP3 negatively regulates the RIP1-induced NF- κ B activation in TLR3 signaling pathway.⁷⁴ Analysis of transgenic mice expressing a kinase dead version of another RIP family member, RIP4, suggested that RIP4 may be required for BCR signaling to NF- κ B.⁸¹ RIP4 was cloned based on its association with PKC β ,⁸² suggesting that the involvement of RIP1 or RIP2 in TCR signaling may allow for the interaction with other PKC isoforms. Biochemical experiments suggested that RIP4 is involved in PKC activation of NF- κ B that is independent of Bcl10.⁸³ However, additional *in vivo* data is needed to confirm the existence of this

Bcl10-independent signaling pathway in NF- κ B activation. A detailed understanding of RIP function awaits further investigation.

Downstream of RIP, TRAF2 and TRAF5 are the mediators of IKK activation in TNF α induced NF- κ B activation signaling pathway. TRAF2 regulates TNF α induced NF- κ B activation in cooperation with TRAF5. TRAF2 and TRAF5 single-knockouts show a mild phenotype, but TRAF2/TRAF5 double knockout are severely impaired in TNF α -induced NF- κ B activation, and are therefore very sensitive to TNF α -induced apoptosis.⁸⁴⁻⁸⁶ How TRAF2/5 and RIP activate IKK and NF- κ B is still controversial.⁹ It was reported that MEKK3 is involved in IKK activation downstream of TRAF2 and RIP in response to TNF α stimulation.⁸⁷ Interestingly, MEKK3 appears to be a multiple edge sword, it is also required for IL-1 and LPS induced activation of NF- κ B, as well as JNK and p38, but not ERK,⁸⁸ perhaps due to the existence of TRAF6, another member of TRAF family in the IL-1 and TLR signaling pathway.

NIK, which is required for activation of the noncanonical NF- κ B pathway by Blys in B cells as discussed above, also plays a role in TCR induced NF- κ B activation, which is distinct from the PKC mediated signaling pathway.⁸⁹ Since NF- κ B activation is only slightly attenuated in NIK mutant thymocytes as well as mature T cells, it appears that NIK may not play a major role in NF- κ B activation in T cells. PKR, the sensor kinase for virus infection and perhaps also involved in LPS stimulated NF- κ B activation,⁹⁰ was reported to directly activate IKK by protein-protein interaction.^{91,92} Using Bone marrow or fetal liver derived macrophages of various knockout mice, Hsu et al validated the TLR 4 pathway in macrophage.⁹³ Consistent with the previous finding, in addition to the role in antiviral infection, they found that PKR is required for macrophage apoptosis after activation of TLR4. However, perhaps due to cell type specificity, PKR is not required for the activation of MAPK p38, JNK, ERK or activation of IKK in response to LPS stimulation in macrophage. This is different from that in embryonic fibroblast where PKR is required for p38 activation in response to LPS and other proinflammatory stimuli.⁹⁰ Hence, the full physiological function of PKR in NF- κ B activation remains to be determined in a variety of cell types.

TAK1 might be the most potential candidate as IKK kinase in multiple signaling pathways, perhaps due to its interaction with TRAF6. Interaction of TAK1 and TRAF6 involves TAK1 binding proteins TAB1 and TAB2. Association of TAK1 with TRAF6 leads to activation of TAK1, and activated TAK1 in turn activates IKK.⁵⁰ However, TAB1 was suggested to be important for heart development.⁹⁴ Analysis of TAB2 knockout mice indicated that the TAK1:TAB complex is not essential for IL-1 and TNF α signaling, but is required for preventing liver apoptosis,⁹⁵ suggesting the existence of other signaling molecules that link TRAF6 and IKK. One candidate of these signaling molecules is TAB3.⁹⁶ TAB3 and TAB2 bind cooperatively, but not competitively, to TRAF6, and TAB3 associates with both TRAF2 and TRAF6 and therefore links them with TAK1. RNA interfering experiment demonstrated that TAB2 and TAB3 play a redundant but critical role in the IL-1- and TNF-induced activation of TAK1.⁹⁶ These finding explained the involvement of TAK1 in both IL-1 and TNF signaling pathway.⁹⁷ If this were the truly physiological situation, it would be interesting to explore the differential role of TAK1 and MEKK3, another MAP3K involved in both IL-1 and TNF induced NF- κ B activation.^{87,88} Much convincing data should be the analysis of TAK1 knockout mice and cells.

The critical role of IKK and many of its upstream signaling molecules in NF- κ B activation has been confirmed by gene targeting experiments. IKK upstream signaling molecules are diverse. A better understanding of NF- κ B activation may provide helpful information for the design of drugs targeting NF- κ B in various diseases such as cancer, inflammation, autoimmune diseases and infectious disease. However, due to the general involvement of NF- κ B in developmental and functional aspects of various cells, cell type-specific inhibition of NF- κ B is needed for meaningful NF- κ B targeted therapies. Attractive targets for such drugs include specific inhibitors for PKC θ in T lymphocytes, PKC β in B lymphocytes, or Bcl10 in T and B cells. On the other hand, transcriptional targets of NF- κ B could be more specific target, and might also represent interesting modalities for drug intervention.

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