Upstream Signal Transduction of NF-kB Activation
(Review)

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Abstract
NF-κB is a transcription factor governing the expression of genes involved in the
immune response, embryo or cell lineage development, cell apoptosis, cell cycle
progression, inflammation, and oncogenesis. During past few years, tremendous
attention has been focused on the upstream signaling pathways leading to the
activation of this transcription factor. Many of these signaling molecules can serve as
potential pharmaceutical targets for the specific inhibition of NF-κB activation and the
subsequent interference of disease processes. However, how these molecules
interact with each other is still a debatable issue. Since many nodal signal molecules
in this pathway relay more than one of the upstream signals to their downstream
targets, it has been speculated that the transmission of signals involves a network,
rather than a linear sequence in the activation of NF-κB. Thus, elucidation of the
detailed relationships among the upstream signaling molecules of NF-κB activation
will be important in developing pharmaceutical inhibitors that specifically inhibit the
activation of NF-κB. Such inhibitors would be predicted to have powerful anti-
inflammatory and/or anti-carcinogenic effects.
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The NF-κB transcription factor was first discovered in 1986. Knowledge of the activation of this transcription factor lagged behind the understanding of its function. Nevertheless, tremendous progress has been achieved over the last two years regarding the signal transduction pathways leading to the activation of NF-κB including the structure and function of IκB kinase complexes (IKK)\textsuperscript{1}, the upstream signaling pathways, the interactions among diverse signaling components, and the extracellular regulators.

At present, five mammalian NF-κB family members have been identified and cloned. These include NF-KB1 NF-κB1 (p50/p105), NF-KB2 NF-κB2 (p52/p100), RelA RelA(p65), RelB RelB, and c-Rel-c-Rel. All these NF-κB family members share a highly conserved Rel homology domain (RHD) responsible for DNA binding, dimerization, and interaction with IκB, the intracellular inhibitor of NF-κB. The C-terminal regions of RelA, RelB and c- Rel contain a transactivating domain that is important for NF-κB-mediated gene transactivation. The C-termini of the precursor molecules for p50 and p52, p105 and p100, contain multiple copies of the so-called ankyrin repeat, which is found in IκB family members, including IκBα, IκBβ, IκBε, Bcl3, and Drosophila cactus.

Diverse stimuli, which typically include cytokines, mitogens, environmental and occupational hazards, toxic metals, intracellular stresses, viral or bacterial products, and UV light, induce expression of early response genes through the NF-κB family of transcription factors. In resting cells, NF-κB is sequestered in the cytoplasm in an inactive form through its association with one of several inhibitory molecules, including IκBα, IκBβ, IκBε, p105, and p100. Activation of the NF-κB signaling cascade results in complete degradation of IκB or partial degradation of the C-termini of p105 and p100 precursors, allowing the translocation of NF-κB to the nucleus, where it induces transcription (Fig. 1). Activated NF-κB binds to the enhancer or promoter regions of target genes and regulates transcription of genes mediating cell-to-cell interaction, intercellular communication, cell recruitment or transmigration, amplification or spreading of primary pathogenic signals, and initiation or acceleration of carcinogenesis. The consensus binding site of NF-κB is composed of the GGGRNNYYCC sequence, where R is purine, Y is pyrimidine, and N is any bases.
Figure 1: Simplified signal transduction pathways of NF-κB activation. Pro-inflammatory signals, mainly TNFα, IL-1 or Toll, bind to their corresponding receptors, leading to a recruitment of receptor-associated proteins, such as MyD88 and IRAK for IL-1R/TLR, TRADD and RIP1 for TNF receptor. In turn these associated proteins recruit TRAF2 or TRAF6, both of which activate TAK1 possibly through a non-destructive G76-K63 polyubiquitin chain-dependent mechanism (Ub63). Activated TAK1 or other MAPKKK family kinases, such as NIK and MEKK1, may phosphorylate and activate IKK complexes that are responsible for the phosphorylation of the IκB protein. Phosphorylated IκB proteins are recognized and modified by the G76-K48 polyubiquitin chain (Ub48) via the SCF-β-TrCP complex. This process is followed by proteasome-mediated degradation of IκBs. Stress signals resulting in the generation of ROIs contribute to the activation of NF-κB and may involve the sequential activation of ASK1, SEK1 and JNK. Activated JNK induces the accumulation of β-TrCP protein, which facilitates the ubiquitination process of IκB proteins.

I. IκB kinase complexes (IKK)
The expanding family of IKK, which includes the IKKα, IKKβ, IKKγ, and IKKι/ε, has, over the past three years, been implicated in the phosphorylation of several IκB proteins and NF-κB family proteins, such as IκBα, IκBβ, IκBγ, IκBε, p105, p100, and RelA(p65) in response to numerous and diverse stimuli. In addition, some non-IκB/NF-κB family proteins, such as β-catenin and HIV vpu protein, have also been implicated as potential IKK substrates. All IκB proteins contain two conserved serine
(S) residues within their N-terminal domain. Phosphorylation of these conserved S residues by IκB kinase (IKK) in response to inducers, leads to the immediate polyubiquitination of IκB proteins by SCF-β-TrCP, a ubiquitin ligase E3 complex. This modification subsequently targets IκB proteins for rapid degradation by the 26S proteasome. The first identified IKK complex, which is also the major IKK complex in most cell types, contains two catalytic subunits, IKKα and IKKβ, and a structural component named NEMO/IKKγ/IKKAP, which may relay upstream signals to IKK and possibly promote assembly of the IKK complex.

IKKα and IKKβ share 50% sequence homology. Both proteins contain a N-terminal kinase domain, a C-terminal region with a leucine zipper, and a helix-loop-helix domain. An activation loop similar to the one found in the MAP-kinase kinase (MEK) family of proteins has been identified between the kinase subdomains VII and VIII of IKKα and IKKβ. Studies by in vitro or ex vivo approaches indicate that both IKKα and IKKβ are interchangeable in phosphorylating S32/S36 of IκBα, and S19/S23 of IκBβ. However, substantial differences in function and regulation of IKKα and IKKβ have been documented. First, IKKβ is far more potent than IKKα in IκBα phosphorylation in response to proinflammatory stimuli, such as the signals induced by TNFα, IL-1 and LPS. Second, whereas IKKα seems to be more responsible for NF-κB-inducing kinase (NIK) signals, IKKβ appears more important in mediating MEKK1 reactions. Third, gene knockout studies demonstrated that IKKα, but not IKKβ, is physiologically involved in NIK-mediated carboxyl terminal phosphorylation and subsequent process of NF-κB2 (p100) precursor. Fourth, IKKα controls keratinocyte differentiation by a kinase-independent mechanism that affects the production of keratinocyte differentiation-inducing factor (kDIF). IKKβ, in contrast, is not necessary for this function. Finally, although both IKKα and IKKβ can phosphorylate multiple regions of β-catenin, an opposite effect of IKKα and IKKβ on the transcriptional activity and intracellular localization of β-catenin has been observed. Using mouse embryo fibroblasts (MEF) lacking IKKα or IKKβ gene, Lamberti et al reported that IKKα increased the nuclear localization and transcriptional activity of β-catenin, whereas IKKβ decreased the nuclear localization and transcriptional activity of β-catenin.

Limited information is available concerning another recently identified IKK complex, IKKι/ε. In contrast to the original IKK complex, this new IKK complex does not contain IKKα, β or γ. IKKι/ε shares 27% homology with IKKα and IKKβ and possibly
mediates NF-κB-activating kinase (NAK) signaling, PMA/PKCε-induced S36 phosphorylation of IκBα, and NF-κB activation. In contrast to IKKα and IKKβ, which are constitutively expressed in most cell types, the expression of IKKi/ε is inducible. In the mouse macrophage cell line, RAW264.7, lipopolysaccharide (LPS) and some other NF-κB-inducing cytokines, can drastically induce the accumulation of IKKi/ε mRNA. Intriguingly, none of these inducers seem to be able to stimulate the kinase activity of transfected IKKi/ε. Yeast two-hybrid screening by Nomura et al. recently showed that the C-terminal portion of IKKi/ε can specifically associate with the N-terminal domain of I-TRAF/TANK, an interaction protein of tumor necrosis factor receptor-associated factor. Thus, it is possible that IKKi/ε either acts further upstream of IKKα/β or at the same hierarchical level of IKKα/β after its association with I-TRAF/TANK.

The predominant role of IKK is its activity as a serine/threonine kinase phosphorylating IκB family proteins. Most of IκB family proteins contain a conserved DSGXXS motif, where X is any amino acid. IKK is also able to phosphorylate NF-κB p65 protein on a non-consensus site, S536. The ability of IKK to exert its profound kinase activity has led many intensive investigations to explore its likely role in other cellular responses. Homology searches of the gene bank protein sequence database reveal that a number of non-IκB/NF-κB family proteins also contain this motif. These proteins include β-catenin, HIV vpu protein, phosphoinositide 3-kinase enhancer (centaurin), c-Ski, Rho/Rac guanine nucleotide exchange factor, and a number of other potential substrates listed in table 1. Except for β-catenin, which has been recently demonstrated to be an IKK substrate, no experimental data so far suggests that IKK can phosphorylate these non-IκB/NF-κB family proteins. New evidence suggests that IKKα controls keratinocyte differentiation and IKKβ attenuates insulin signaling related to type 2 diabetes and obesity. Thus, in addition to NF-κB signaling, IKK might be involved in several other cellular signal transduction pathways in either a kinase activity-dependent or a kinase activity-independent manner.

There are several good candidates for the inhibition of IKK activity. One group is the nonsteroidal anti-inflammatory drugs (NSAIDs), including aspirin and sodium salicylate. NSAIDs have been previously shown to inhibit activation of NF-κB and cytokine-induced mRNA of cell adhesion molecules. Later studies suggested that these effects of NSAIDs are the result of specific inhibition of ATP-binding to IKKβ,
which is independent of their cyclooxygenase-2 (COX-2) inhibitory activity. A second candidate is the 15d-PGJ$_2$, an anti-inflammatory cyclopentenone prostaglandin and a natural ligand for peroxisome proliferator-activated receptor $\gamma$ (PPAR$\gamma$). In Jurkat T cells or HeLa cells, 15d-PGJ$_2$ inhibits TPA- or TNF$\alpha$-induced NF-$\kappa$B activation in a PPAR$\gamma$-independent manner. A direct modification of cysteine 179 (C179) in the activation loop of IKK$\beta$ by 15d-PGJ$_2$ was observed in an \textit{in vitro} IKK kinase activity assay. The IC$_{50}$ for inhibition of IKK activity was around 5 $\mu$M. The same concentration of 15d-PGJ$_2$, in contrast, stimulated JNK activity, indicating that 15d-PGJ$_2$ or its analogs may have therapeutic potential for diseases in which inhibition of IKK and NF-$\kappa$B may be desirable. However, it should be noted that the inhibitory effect of 15d-PGJ$_2$ on IKK and NF-$\kappa$B might be cell type or stimulus dependent. This becomes evident by the fact that 15d-PGJ$_2$ potentiated LPS-induced gene expression of IL-8, a NF-$\kappa$B targeting gene. The third group of IKK inhibitors includes several plant extracts that have been shown to reduce IKK activity in some experimental systems. These extracts include resveratrol, parthenolide, and green tea polyphenol (-)-epigallocatechin-3-gallate. The specificity and potential application of these natural products in inhibiting IKK activity remain to be investigated. Finally, two relatively specific IKK inhibitors are being developed by Signal Pharmaceuticals and Novartis Pharma AG, respectively. Signal Pharmaceuticals developed a selective IKK$\beta$ inhibitor named SPC839 that inhibits IKK$\beta$ with nanomolar potency and IKK$\alpha$ with micromolar potency.

II. Upstream kinases of IKK

1. MEKK1

MEKK1 is a mammalian serine/threonine kinase in the mitogen-activated protein kinase kinase kinase (MAPKKK) group. It was found that MEKK1 was a far more important activator for JNK signaling rather than ERK signaling as was proposed originally. The first evidence indicating the involvement of MEKK1 in signal-induced IKK activation was provided by Lee \textit{et al.}. In their studies, they reported that the addition of the recombinant catalytic domain of MEKK1 (MEKK1$\Delta$) to the partially enriched fraction of nonstimulated HeLa cells stimulated an IKK-like kinase activity that phosphorylated IkB$\alpha$ at S32 and S36 and subsequent ubiquitination and degradation of IkB$\alpha$. Follow-up studies demonstrated that overexpression of MEKK1
stimulated the NF-κB-dependent transcriptional reporter. The activation of NF-κB by HTLV Tax protein was shown to require MEKK1. MEKK1 may also contribute to Toll- and IL-1 receptor-mediated IKK activation, as demonstrated by an adaptor protein, known as evolutionarily conserved signaling intermediate in Toll pathways (ECSIT) that could promote the proteolytic activation of MEKK1 and subsequent activation of NF-κB. Further studies by Mercurio et al. indicated the presence of a protein in the IKK complex that is recognized by anti-MEKK1 antiserum.

Although MEKK1 has been shown to contribute to IKK activation in a number of studies, the precise molecular link between these two kinases remains unclear. One intriguing possibility is that MEKK1 may directly phosphorylate both IKKα and IKKβ at the MAPKK activation loop, 176/177 S-X-X-X-S 180/181, where X is any amino acid. Substitution of these serines with alanine residues inactivates both kinases, whereas phosphomimetic glutamic acid substitution at these positions results in constitutively active kinases. Nevertheless, it remains to be confirmed whether MEKK1 is a physiological activator of IKK in cells in response to various stimuli. Indeed, a recent study by Xia et al. demonstrated that inactivation of MEKK1 did not result in an impairment of NF-κB activation in response to TNFα, IL-1, LPS, and dsRNA.

2. NF-κB-Inducing Kinase (NIK)

NIK, a member of the MAPKKK family, was originally identified as a tumor necrosis factor (TNFα) receptor associating factor 2 (TRAF2)-interacting kinase whose overexpression results in potent NF-κB activation without any considerable effect on MAPKs. A study using yeast two-hybrid screens identified an interaction between NIK and IKK, suggesting that NIK might be a direct upstream activator of IKK. Transient transfection of NIK into human embryonic kidney 293 cells indicated that IKKα was more responsive to NIK, whereas IKKβ was slightly more responsive to MEKK1. When the abilities of MEKK1 and NIK to activate total IKK kinase activity are compared, most of studies show that NIK is a much stronger activator of the NF-κB transcriptional reporter than MEKK1. NIK could preferentially phosphorylate IKKα on S176 in the activation loop, leading to the activation of IKKα kinase activity. In contrast, MEKK1 was found to preferentially phosphorylate the corresponding serine residue, S177, in the activation loop of IKKβ. A dominant negative mutant of NIK blocked NF-κB activation by TNFα, interleukin-1 (IL-1), FasFas, Toll-like receptors 2 and 4, LMP1 and CD3/CD28 stimulation. Thus, NIK appears to be a general kinase.
mediating IKK activation induced by diverse stimuli. However, a recent analysis using the NIK-mutant mouse strain *alyymphoplasia (aly)* contradicted this assumption. The *Alymphoplasia* mouse strain failed to develop lymphoid organs, such as lymph nodes and Peyer’s patches due to a point mutation in the NIK locus. The mutation of NIK locus results in disruption of interactions between NIK and IKKα or TRAF proteins. Further analysis indicated that the *aly* mutation did not affect TNFα-induced activation of NF-κB but only blocked lymphotoxin-mediated activation of NF-κB. Similarly, studies using cells derived from NIK-deficient mice have indicated that NIK appears to be dispensable in IKK activation induced by TNFα or IL-1. Intriguingly, lymphotoxin β induced a normal NF-κB DNA-binding activity in NIK-deficient cells, whereas the same treatment failed to induce NF-κB reporter gene activity or NF-κB target gene expression. It raises the possibility that NIK may be specifically involved in IKK activation induced by lymphotoxin but not others.

3. NF-κB activating kinase (NAK)

Several groups independently identified a novel serine/threonine kinase, possibly activating IKK through direct phosphorylation in cells stimulated with PMA. This novel kinase was named NAK, TANK-binding-kinase 1 (TBK1), or T2K. Pomerantz and Baltimore cloned NAK by a yeast two-hybrid screen using the N-terminal stimulatory domain of TANK 1-190 fused to GAL4 as bait, and a human B-cell library fused to the GAL4 activation domain. The same kinase was also identified by PCR using degenerate primers based on sequences common to IKKα and IKKβ. The amino acid sequence analysis indicated that the NAK protein contains a kinase domain at its N-terminus that exhibits about 30% identity to the corresponding kinase domains of IKKα and IKKβ, and more than 60% identity to the corresponding kinase domain of IKKi/ε. The report by Pomerantz and Baltimore showed that NAK might form a ternary complex with TRAF-2TANK and TRAF-2, suggesting that NAK functions to the far more upstream of the signal cascade leading to IKK activation, whereas *in vitro* kinase activation assay by Tojima and co-workers demonstrated that NAK was a direct upstream kinase phosphorylating IKKβ. Interestingly, activation of endogenous NAK resulted in only S36, but not S32 phosphorylation of IkBα, a similar phenomenon observed in recombinant IKKi/ε-mediated IkBα phosphorylation. Since both IKKα and IKKβ are able to phosphorylate both S32 and S36 of IkBα protein, it is unclear whether IKKi/ε or a novel IKK isozyme functions as a downstream kinase of
NAK to induce S36 phosphorylation of IκBα. Transient transfection studies showed that dominant negative NAK inhibited NF-κB transcriptional reporter activity induced by PMA, PKCε, and PDGF, but not by TNFα, IL-1β, LPS, or ionizing radiation. These results, therefore, suggest that NAK is likely to be a downstream kinase of PKCε or related isozymes, and an upstream kinase of IKK in the signaling pathway through which growth factors, such as PDGF, stimulate NF-κB activity.

4. Akt (PKB)
The pro-survival function of Akt has been well documented. The kinase activity of Akt is activated via the phosphoinositide-3-OH kinase (PI3K) and P13K-dependent kinase 1/2 (PDK1/2) signaling pathways. Overexpression or constitutive activation of Akt has been associated with tumorigenesis in a number of studies. As a serine/threonine kinase, Akt is able to phosphorylate the pro-apoptotic protein Bad, the anti-apoptotic protein Bcl-x, the apoptotic protease caspase-9, the Forkhead transcription factors, and eNOS. However, considerable controversy remains regarding the involvement of Akt in signal-induced IKK activation. Studies by Ozes et al. and Xie et al. indicated that Akt was required for TNFα- or G protein activator-induced NF-κB activation by directly phosphorylating and activating IKKα in 293, HeLa, and ME-180 cells. A putative Akt phosphorylation site at amino acids 18 to 23 in both IKKα and IKKβ was identified. Akt induced T23 phosphorylation of IKKα both in vitro and in vivo. Mutation of T23 significantly decreased Akt-induced IKKα phosphorylation and TNFα-induced NF-κB activation in 293, HeLa, and ME-180 cells. By contrast, Romashkova et al. demonstrated that Akt was involved in PDGF-mediated, but not TNFα- or PMA-mediated NF-κB activation in human or rat fibroblasts. In this study, the authors suggested that upon PDGF stimulation, Akt could transiently associate with IKK and induce the activation IKK, especially IKKβ. Several other studies, however, contradicting these reports, suggested that the effects of Akt on NF-κB did not occur at the level of IKK activation in several cell types. A study by Delhase and co-workers indicated that Akt activation induced by IGF-I failed to activate IKKα, IκBα phosphorylation and degradation, or NF-κB DNA binding in HeLa cells, the same cell line used by Ozes et al. Similarly, several recent studies showed that Akt was not involved in TNFα-induced NF-κB activation in human vascular smooth muscle cells, skin fibroblasts, or endothelial cells. Rather, Akt might enhance the ability of the p65 (RelA) transactivation to induce transcription
In Jurkat T-cells, Akt alone failed to activate NF-κB, but it was capable of potentiating NF-κB activation induced by PMA, partially by enhancing IκBβ degradation. Evidence further supporting this notion is the observation that expression of constitutively active Akt upregulates the mRNA level of β-TrCP, a subunit of the SCF-β-TrCP complex responsible for the ubiquitination of IκBα or IκBβ proteins. Thus, it is possible that Akt phosphorylates IKK in a cell context- and stimulation-dependent manner. It is also highly possible that several different mechanisms are involved in Akt-regulated NF-κB activation. One remaining question is whether or not upstream kinases of Akt, such as PDK1 and PDK2, also activate IKK, since both IKKα and IKKβ contain a putative PDK1 phosphorylation site (S-F-X-G-T-X-X-Y-X-A-P-E) directly juxtaposed to the MAPKKK phosphorylation site.

5. Mixed-Lineage Kinase 3 (MLK3)

MLK3, another member of the MAPKKK family, contains an N-terminal SH3 domain, followed by the catalytic domain and two tandem leucine/isoleucine zippers, a basic region, a Cdc42/Rac binding motif, and a proline-rich C terminus. Based on these structural characteristics, MLK3 may associate with a variety of protein modules. Studies by Hehner et al. suggested that MLK3 could directly associate with IKK complex through its leucine zipper domain and phosphorylate Sl76 of IKKα and Sl77 and Sl81 of IKKβ. Transfection of Jurkat T cells with a kinase-mutated form of MLK3 blocked CD3-CD28 signal- and PMA-induced NF-κB transcriptional activity. No significant influence of this mutated MLK3 was observed on either TNFα- or IL-1-induced NF-κB activation. These results suggest that MLK3 may be important in mediating T-cell co-stimulation-induced activation of IKK and consequent NF-κB-dependent transcription. MLK3 has also been shown to form a complex with a JNK scaffold protein JIP and stimulate JNK activation. Thus, MLK3 may function as an integral molecule between the signaling pathways leading to the activation of NF-κB and JNK, which would provide a molecular explanation why many stimuli induce NF-κB and JNK simultaneously under certain circumstances.

6. TGFβ-Activated Kinase 1 (TAK1)

TAK1 is a member of the MAPKKK (MAP3K) family, which was originally identified as a kinase mediating the signaling pathway of TGFβ superfamily members. Transfection of the cells with an activated form of TAK1, in which the N-terminal 22 amino acids are deleted, induces expression of a reporter gene governed by TGFβ-
responsive promoter. However, the biochemical link between TGFβ and TAK1 has been elusive. Ironically, the contributions of TAK1 to signal-induced NF-κB and JNK activation have been studied intensively. Several new insights into the roles of TAK1 in IKK activation have emerged in the cellular response to cytokines or Toll signals. The first evidence to suggest that TAK1 is involved in NF-κB signaling came from studies in which overexpression of TAK1 together with its activator protein, TAK1 binding protein 1 (TAB1), induced the nuclear translocation of NF-κB in a NIK-independent manner. Further studies indicated a direct physiological interaction between TAK1 and IKK in unstimulated cells. Recruitment of TAB1 and/or TAB2 to TAK1 activates the kinase activity of TAK1, resulting in phosphorylation of the serine residues in the activation loop of IKK and subsequent dissociation of TAK1 from IKK complex.

In *Drosophila*, a null mutation in the TAK1 gene produces phenotypes similar to that of mutations in immune deficiency (*imd*), and IKK, suggesting that TAK1 is a direct kinase mediating *imd* signals. Genetic studies and sequential chromatographic purification by Wang et al. demonstrate that the kinase activity of TAK1 in response to IL-1 is dependent on the TRAF6 protein, which has been modified by a distinct polyubiquitin chain assembled through the lysine 63 (K63) at each ubiquitin molecules. In contrast to the polyubiquitin chain in which the C-terminal glycine 76 (G76) of one ubiquitin is ligated to the K48 side chain of the neighboring ubiquitin, the polyubiquitin chain linked through G76-K63 does not target proteins for proteasomal degradation, but rather, activates the function of proteins (see below). The major debatable issues in TAK1-induced IKK activation are the involvement and hierarchical position of NIK. Whereas several reports clearly suggest that NIK is not involved in TAK1-induced IKK activation and TAK1 is a direct upstream kinase phosphorylating IKK in HeLa cells treated with IL-1, Ninomiya-Tsuji et al. showed that NIK is a mediator of TAK1-induced IKK activation in IL-1-treated 293 cells. It is unclear whether this discrepancy is due to the cell type or subtle differences in overexpression of the dominant-negative inactive NIK mutant.

### 7. Other Kinases

A variety of other kinases have been reported to function upstream of IKK. Because of the lack of evidence of direct association of these kinases with IKK upon activation or specific phosphorylation site(s) of these kinases on IKK, it is unclear whether these kinases are direct upstream kinases phosphorylating and activating of IKK, or far
more distal kinases indirectly activating IKK. These kinases include Cot, PKCζ, PKCα, PKCθ, or PKR etc. In light of the fact that a variety of kinases can affect IKK, it seems likely that different cell types and stimuli may utilize distinct upstream kinases for the activation of IKK. An example to support this notion is the observation that PKCθ and Cot kinase participate in CD3-CD28 costimulation signal-induced, but not TNFα-induced, activation of NF-κB.

III. Mechanisms of Ubiquitination in NF-κB Activation

As detailed above, the activation of NF-κB by most of the extracellular inducers is dependent on the phosphorylation and subsequent degradation of IκB proteins. A crucial step during this process is the phosphorylation-dependent conjugation of IκB proteins with polyubiquitin chain, a marker required for the proteasomal degradation of IκBα. Whereas the ubiquitination sites on IκBβ and IκBε have not been definitely identified, lysines 21 and 22 (K21 and K22) on the IκBα protein were considered as the major sites conjugated by the polyubiquitin chain.

**Figure 2**: SCF-β-TRCP ubiquitin ligase complex-mediated ubiquitination of IκB proteins. The basic components of this E3 complex include Skp1, Cul-1 (CDC53), and the F-box protein, β-TRCP. β-TRCP recognizes and links the phosphorylated IκB proteins to this complex allowing the ubiquitination of IκBs by ubiquitin-conjugating enzyme E2 following the C-terminal G residue activation of ubiquitin by ubiquitin-activating enzyme E1.

Ubiquitin is a highly conserved and heat stable 76-amino acid protein found in virtually all types of eukaryotic cells. Ubiquitination of proteins involves three or four sequential steps (Fig. 2). Initially, the C-terminal glycine (G76) of ubiquitin is activated by ATP to form a high energy thiolester intermediate catalyzed by the ubiquitin-activating enzyme (Uba or E1). Activated ubiquitin is then transferred from E1 to one of many distinct ubiquitin-conjugating enzymes (Ubc or E2), forming a similar thioester-linked complex. Finally, with the aid of ubiquitin ligases (E3), an isopeptide bond is formed between the activated C-terminal G76 of ubiquitin and an ε-NH₂.
group of a K residue of the substrate. In successive reactions, polyubiquitin chain, is synthesized by progressive transfer of ubiquitin moieties to K48 or K63 of the previously conjugated ubiquitin molecule, forming G76-K48 or G76-K63 isopeptide bonds. An assembly factor, named Ufd or E4, may be required for this process. The specificity of protein ubiquitination is usually determined by the ubiquitin ligase E3 that recognizes specific substrates. At least three types of ubiquitin ligase E3 complexes have been well documented. These ligase complexes include the Skp1-cullin-F-box (SCF) complex, the VHL protein-elongin B-elongin complex (VBC), and the anaphase promoting complex (APC). The ubiquitin ligase E3, responsible for the ubiquitination of IκBα, is the SCF complex containing a F-box/Trp-Asp repeating (WD) protein named β-TrCP (Fig. 2). Following phosphorylation of S32 and S36 in the conserved DSGXXS motif of IκBα by IKK, the β-TrCP subunit of the SCF complex recognizes and binds to the phosphorylated DSGXXS motif of IκBα. The binding of SCF to IκBα results in the association of SCF with specific E2s, including Ubc3, Ubc4, Ubc5, and Ubc9. These E2s are able to catalyze the ubiquitin conjugation of IκBα and the assembly of G76-K48 polyubiquitin chain. Consistent with the likely role for SCF-β-TrCP as a ubiquitin ligase complex conjugating polyubiquitin chain to IκBα, is the observation that Slimb protein, a Drosophila homology of mammalian β-TrCP, is required for the ubiquitination of Cactus, an IκB-like protein inhibiting the activation of the Drosophila NF-κB homolog, Dorsal. During dorsoventral patterning of the early Drosophila embryo, the Dorsal protein is activated specifically on the ventral side of the embryo by the Toll receptor-signaling pathway. These findings point to the existence of an evolutionarily conserved pathway for specific ubiquitination of the IκBα protein for the purpose of dynamic signal transduction from the receptor to NF-κB.

In parallel studies of signal-induced IκBα ubiquitination, several reports indicated that this process could be antagonized by SUMO-1 (small ubiquitin-related modifier-1) modification of IκBα on the same residues where the polyubiquitin chain is conjugated, or by unknown product(s) of nonpathogenic Salmonella bacteria. SUMO-1 is one of the best-characterized members of ubiquitin-related proteins. Conjugation of SUMO-1 to substrates requires SUMO-1-activating enzyme Aos/Uba2, and SUMO-1-conjugating enzyme, Ubc9. Although substrates can be modified by several SUMO-1 at distinct sites, no multi-SUMO-1 chains are apparently
formed. In contrast to ubiquitination of \( \kappa B \alpha \) protein, SUMO-1 conjugation does not target \( \kappa B \alpha \) to proteasomal degradation. The inhibition of NF-\( \kappa B \) by certain bacterial pathogens may be through a mechanism affecting the conjugation of SUMO-1 on \( \kappa B \) proteins. One example is the observation that \( \text{YopJ} \), a protein product encoded by a 70-kB plasmid harbored in the \textit{Yersinia} species that caused the Black Death in the Middle Ages, inhibits MEKK1-induced NF-\( \kappa B \) activation. Earlier study by Orth et al. indicated that the inhibition of NF-\( \kappa B \) by \( \text{YopJ} \) is through direct interaction of \( \text{YopJ} \) with IKK\( \beta \) but not with IKK\( \alpha \). Structural analysis of \( \text{YopJ} \) protein by the same group later suggested that \( \text{YopJ} \) might be a SUMO-protease promoting the conversion of precursor SUMO-1 to mature SUMO-1. Nevertheless, whether \( \text{YopJ} \) enhances the conjugation of SUMO-1 on \( \kappa B \alpha \) has not been demonstrated.

The vast majority of ubiquitination reactions in which the proteins are ubiquitinated via G76-K48 assembly of the polyubiquitin chain target protein for proteasomal degradation. Examples include the ubiquitination of \( \kappa B \alpha \), p53, cyclins, c- Jun, and others. This is not, however, the case of ubiquitination of proteins via G76-K63 assembly of polyubiquitin chain. The biochemical evidence of G76-K63 assembly of the polyubiquitin chain remains elusive, but it appears to be independent of proteasomal degradation. Recent studies by Wang et al. suggest that linkage of the G76-K63 polyubiquitin chain with TRAF6 protein plays an important role in mediating TLR/IL-1R signal-induced activation of TAK1, an upstream kinase of IKK. TRAF6 itself exhibits the ubiquitin ligase E3 activity by the structural characteristic of RING fingers in its C-terminus.

The nature of the upstream regulators that promote G76-K63 ubiquitination of TRAF6 is less clear. One good candidate, however, is the Ubc complex composed of Ubc13, a member of the ubiquitin-conjugating enzyme E2 family, and Uev1A, a ubiquitin-conjugating E2 enzyme variant. In yeast and mammalian cells, both Ubc13 and Uev1A are considered the major enzymes required for the synthesis of G76-K63 polyubiquitin. In chromatographic purification of HeLa cell cytoplasmic extracts, the Ubc13/Uev1A complex was found to be co-eluted with TRAF6 and appeared to be essential in TRAF6-induced TAK1 and subsequent IKK activation. However, in \textit{Drosophila}, this Ubc13/Uev1A-induced K63 polyubiquitination of TRAF6 has yet to be established, despite the identification of the \textit{Drosophila} homologs of Ubc13 and Uev1A, \textit{bendless} and \textit{dUev1A}, respectively. Interestingly, Ubc13/Uev1A was also
found to interact genetically with a DNA repair protein, Rad5, indicating that it is likely to be coupled to a number of cellular processes. Such a finding supports the likelihood that the activation of the Ubc complex provides a mechanism by which IKK signals can be selectively activated during cellular damage response in vivo.

IV. ROIs: Critical Mediators or bystanders in NF-κB Activation?

Oxidative stress is a hallmark of pathophysiological response resulting from the alterations of cellular redox homoeostasis due to either an over-production of reactive oxygen intermediates (ROIs) or a deficiency in buffering or scavenging system for ROIs. Typically, the oxidatively stressed cells exhibit damage of their macromolecules leading to lipid peroxidation, oxidation of amino acid side chains (especially cysteine), DNA damage, stress response kinase activation and gene expression associated with cell cycle arrest and/or cell apoptosis. Moderate oxidative stress without severe damage of structural and functional macromolecules can be recovered due to the activation of cellular defense systems including nonenzymatic and enzymatic antioxidants. A number of stress response genes are induced to protect cells from the oxidative stress or to repair the ROI-mediated damages. A sustained oxidative stress produced during chronic or acute inflammatory response and/or environmental toxicant exposure, however, will be cytotoxic.

Among all the known oxidative stress inducers, H$_2$O$_2$ and some environmental toxic metals or particles are perhaps the most potent and well studied. Many other agents, such as TNFα, IL-1 and bacterial or viral proteins, also induce oxidative stress. Since the discovery of NF-κB, hundreds of reports have indicated that some extracellular stimuli that induce oxidative stress also activate NF-κB. Thus, it is not too surprising that many researchers attributed a role for ROIs in signal-induced NF-κB activation. Some even proposed that ROIs might be universal molecules mediating the activation of NF-κB in response to a broad range of stimuli. However, the conclusion that ROIs mediate NF-κB activation has been strongly challenged. First, correlations between ROI generation and NF-κB activation do not necessarily mean ROIs are essential mediators linking upstream signals to NF-κB activation. Under certain circumstances, ROI generation may be simply a bystander signal or a secondary response to NF-κB activation. Second, caution should be exercised in interpreting the inhibitory effects of a various antioxidants on signal-induced NF-κB. Many antioxidants can disturb the normal cellular redox status that maintains the
basal signaling potential required for the activation of NF-κB or other intracellular biochemical events even under the non-oxidative stress conditions. In addition, many low-molecular-weight antioxidants may inhibit NF-κB by non-antioxidant actions. Third, it should be noted that several studies show that ROIs fail to activate NF-κB in many experimental systems. Finally, emerging evidence suggests that the DNA binding activity of activated and nuclear translocated NF-κB requires reducing conditions. Oxidation or nitrosylation of the cysteine residue in the DNA binding domain of the NF-κB p50 subunit suppresses the DNA binding and transcriptional activity of NF-κB.

The signal transduction pathway, such as the upstream and proximal kinases, leading to the activation of NF-κB by TNF, IL-1, Toll, LPS, and CD28, has been clearly identified. However, only limited information is available to suggest the responsiveness of these kinases to ROIs. The evidence to implicate ROIs as stimulators of IKK is based on the elevated IKK activity in human epithelial cells or mouse fibroblast cells by the H₂O₂ treatment. In our own studies, we found a modest induction of IKK activity in cellular response to chromium(VI), a potent intracellular H₂O₂ inducer (Chen et al., unpublished). Nevertheless, studies challenging this observation exist. Li and Karin could not detect IKK kinase activity in HeLa cells stimulated with UV-C, another intracellular H₂O₂ inducer, despite the fact that UV-C induced IκBα degradation and NF-κB DNA binding. Similarly, Korn et al. described that H₂O₂ itself failed to stimulate IKK, but rather, inhibited TNFα-induced IKK activity. It is highly likely that H₂O₂ inactivates IKK through direct oxidation of a conserved cysteine 179 (C179) in the kinase domain of IKKβ, a mechanism similar to the inactivation of IKKβ by 15d-PGJ2 and a high concentration of arsenic.
Oxidation of the C-X-X-C motif of Trx induces its dissociation from ASK1, thereby allowing the dimerization and activation of ASK1. JNK is activated by SEK1 that has been phosphorylated by ASK1, leading to the accumulation of β-TrCP which is required for the processes of IκB ubiquitination.

Whereas IKK seems to be a less favorable target-point in ROI-modulated NF-κB activation, kinases other than IKK may serve as bridge molecules linking ROIs to the activating signals of NF-κB. One such kinase, JNK, merits special attention, not only because of its unequivocal activation in response to ROIs, but also because of its potential link to the ubiquitination and subsequent degradation of IκBα. The activation of JNK by ROIs appears to be mediated by the activation of ASK1, a member of the MAPKKK family that phosphorylates and activates SEK1 (MKK4), an upstream kinase of JNK (Fig. 3). In resting cells, ASK1 binds with high affinity to the reduced form of thioredoxin (Trx) which serves as an inhibitor of ASK1 by preventing the dimerization of ASK1. Oxidation of the C-X-X-C motif of Trx by ROIs induces the dissociation of Trx from ASK1, thereby allowing the dimerization of ASK1 and the consequent activation of JNK. We have previously reported that inhibition of JNK by overexpression of a dominant negative SEK1 impaired the degradation of IκBα and the activation of NF-κB induced by vanadate. This observation was further substantiated by Spiegelman et al. who provided convincing evidence indicating the contribution of JNK to the signal-induced ubiquitination of IκBα protein. Activation of JNK resulted in accumulation of β-TrCP, a subunit of the SCF-β-TrCP complex that
recognizes the phosphorylates DSGXXS motif within the \( \kappa B_{\alpha} \) protein and causes the subsequent ubiquitination. While JNK has been implicated in the stabilization of a number of short-lived mRNAs in response to stress, it is plausible to speculate that the JNK-mediated accumulation of \( \beta -\text{TrCP} \) is through stabilization of the \( \beta -\text{TrCP} \) mRNA. Indeed, analysis of the \( \beta -\text{TrCP} \) mRNA sequence by Spiegelman et al. revealed a closely resembled JNK response element in addition to two AU-rich elements in the 3'-UTR region of \( \beta -\text{TrCP} \) mRNA. Since ubiquitination of the \( \kappa B_{\alpha} \) protein is potentially a rate-limiting step, the abundance of \( \beta -\text{TrCP} \) regulated by JNK may serve as an important point of regulation in ROI-induced NF-\( \kappa B \) activation.

**Table 1:** Confirmed and putative IKK substrates in mammalian cells.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Consensus sites</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Confirmed substrates:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IkBa</td>
<td>31 DSGLDSS</td>
<td>3</td>
</tr>
<tr>
<td>IkBb</td>
<td>18 DSGLGS</td>
<td>3</td>
</tr>
<tr>
<td>IkBe</td>
<td>17 DSGIES</td>
<td>3</td>
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<tr>
<td></td>
<td>70 DSTYGS</td>
<td></td>
</tr>
<tr>
<td>p105</td>
<td>921 DSVCDS</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>925 DSGVET</td>
<td>23</td>
</tr>
<tr>
<td>p65</td>
<td>535 SSIAM</td>
<td>24</td>
</tr>
<tr>
<td>b-Catenin</td>
<td>32 DSGIHSS</td>
<td>10</td>
</tr>
<tr>
<td><strong>Putative substrates:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rho-GEF</td>
<td>220 DSGLDSS</td>
<td></td>
</tr>
<tr>
<td>CDC2-related kinase 7</td>
<td>439 DSGLES</td>
<td></td>
</tr>
<tr>
<td>Centaurin b2</td>
<td>386 DSGNES</td>
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<tr>
<td>HNF-3a</td>
<td>35 NSGLGS</td>
<td></td>
</tr>
<tr>
<td>Metastasis suppressor protein</td>
<td>321 DSGFIS</td>
<td></td>
</tr>
<tr>
<td>PPARg coactivator 1</td>
<td>60 DSGFVS</td>
<td></td>
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<tr>
<td>TRAF6</td>
<td>285 DSGYIS</td>
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<tr>
<td>hIRS-1</td>
<td>423 DGFISS</td>
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<tr>
<td>Plakoglobin</td>
<td>23 DSGIHSS</td>
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</tr>
<tr>
<td>Carboxypeptidase A1</td>
<td>175 DGFISS</td>
<td></td>
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</tbody>
</table>
Summary

Increasing evidence implicates dysregulation of signaling pathways leading to the activation of the NF-κB transcription factor in the pathology of various diseases, including autoimmune diseases, neurodegenerative diseases, inflammation, and cancers. Moreover, several human diseases due to the inherited mutations in the genes encoding NF-κB signaling molecules have been recently described. The signal transduction pathways of NF-κB activation therefore represent potential targets for therapeutic intervention. As discussed above, tremendous advances have been made in our understanding of the upstream signaling pathways of NF-κB activation. Yet this understanding is not complete. A fundamental goal for future studies is to focus on the structural and functional aspects of the participating components in these pathways. It seems likely that new aspects of NF-κB signaling will be discovered through such studies.

Abbreviations:

IKK, IκB kinase complex; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinases; ROIs, reactive oxygen intermediates; NIK, NF-κB inducing kinase; NAK, NF-κB activating kinase; TAK1, TGF-β activating kinase 1; MLK, mixed lineage kinase.

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