Upstream Signal Transduction of NF-κB Activation

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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 the past few years, considerable attention has been paid to the upstream signaling pathways that lead to the activation of NF-κB. Many of these signaling molecules can serve as potential pharmaceutical targets for the specific inhibition of NF-κB activation leading to interruption of disease processes. How these molecules interact with each other is however, still a debatable issue. Since many of the signal molecules in this pathway relay more than one of the upstream signals to downstream targets, it has been suggested that the transmission of signals involves a network, rather than a linear sequence in the activation of NF-κB. Thus, the detailed elucidation of the upstream signaling molecules involved with NF-κB activation will be important to the development of pharmaceutical inhibitors that specifically inhibit the activation of NF-κB. Such inhibitors would be predicted to have potent anti-inflammatory and/or anti-carcinogenic effects.

Key words: IKK, kinases, NF-κB, ROIs, signal transduction, ubiquitination.

The NF-κB transcription factor was first discovered in 1986 [1]. Knowledge of how this transcription factor is activated lagged behind our understanding of its function. Nevertheless, tremendous progress has been made over the last two years regarding our understanding of the signal transduction pathways that lead to the activation of NF-κB. This includes the structure and function of IκB kinase complexes (IKK)[1], the upstream signaling pathways, the interactions among diverse signaling components, and the extracellular regulators that control activation of NF-κB [2,3].

At the present time, five mammalian NF-κB family members have been identified and cloned [4,5]. These include NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA(p65), RelB, and c-Rel. All of these NF-κB family members share a highly conserved Rel homology domain (RHD) that is responsible for DNA binding, dimerization, and interaction with IκB, the intracellular inhibitor of NF-κB[6]. 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-terminus of the precursor molecules for p50 and p52, p105 and p100, contain multiple copies of the so-called ankyrin repeat element, which is found in IκB family members, including IκBα, IκBβ, IκBε, Bcl3, and *Drosophila* cactus.

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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 [3-5]. In resting cells, NF-κB is sequestered in the cytoplasm in an inactive form through its association with one of the several inhibitory molecules, including IκBα, IκBβ, IκBε, p105, and p100. Activation of the NF-κB signaling cascade results in the complete degradation of IκB or partial degradation of the C-terminus of p105 and p100 precursors, allowing for 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 that mediate 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 base.

I. IκB Kinase Complexes (IKK)

The expanding family of IKK members, which includes IKKα, IKKβ, IKKγ, and IKKi/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 [3,7-9]. In addition, some non-IκB/NF-κB family proteins, such as β-catenin and HIV vpu protein, have also been implicated as potential IKK

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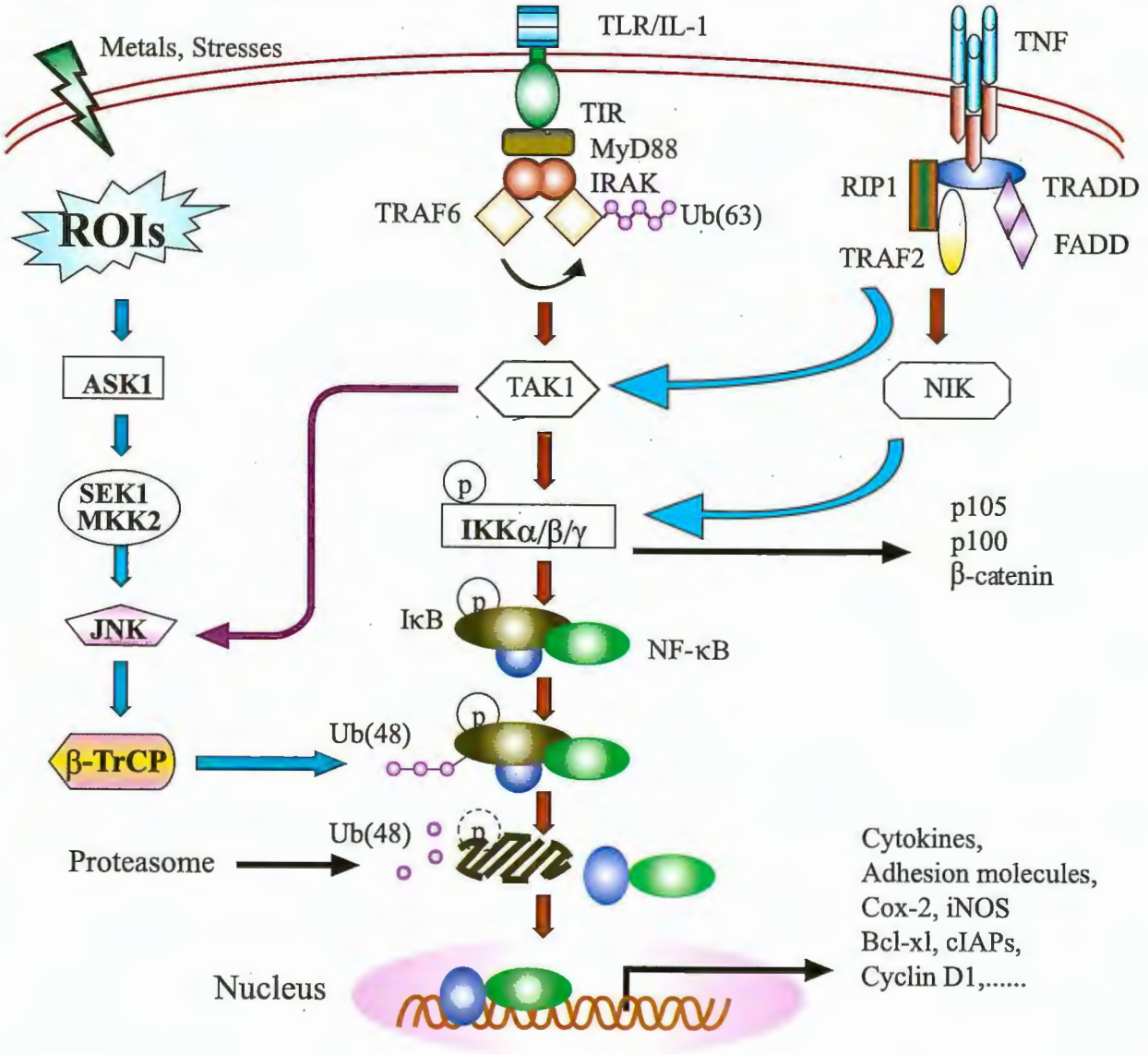

Fig. (1). Simplified signal transduction pathways of NF-κB activation. Pro-inflammatory signals, mainly TNFα, IL-1 or Toll, bind to their corresponding receptors, leading to a recruitment of receptor-associated proteins, such as MyD88 and IRAK for IL-1R/TLR, TRADD and RIP1 for TNF receptor. In turn these 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.

substrates [10]. 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 [11]. This modification subsequently targets IκB proteins for rapid degradation by the 26S proteasome [2-4]. 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 [12-14].

IKKα and IKKβ share 50% sequence homology. Both proteins contain an N-terminal kinase domain, a C-terminal region with a leucine zipper, and a helix-loop-helix domain [12-14]. 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 using *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 [3]. However, substantial differences in the function and regulation of IKKα and IKKβ have been documented. Firstly, IKKβ is far more potent than IKKα in the phosphorylation of IκBα in response to stimuli, brought on by proinflammatory factors such as TNFα, IL-1 and LPS [3]. Secondly, whereas IKKα seems to be more responsible for NF-κB-inducing kinase (NIK) signals, IKKβ appears more important in the mediation of MEKK1 reactions [15]. Thirdly, gene knockout studies have demonstrated that IKKα, but not IKKβ, is physiologically involved in NIK-mediated carboxyl terminal phosphorylation and the subsequent process of the NF-κB2 (p100) precursor [16]. Fourthly, IKKα controls keratinocyte differentiation by a kinase-independent mechanism that affects the production of the keratinocyte differentiation-inducing factor (kDIF) [17]. 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 either the IKKα or IKKβ gene, Lamberti *et al.* [10] 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, IKKi/ε. In contrast to the original IKK complex, this new IKK complex does not contain IKKα, β or γ [18-20]. IKKi/ε shares 27% homology with IKKα and IKKβ and is believed to mediate NF-κB-activating kinase (NAK) signaling, PMA/PKCε-induced S36 phosphorylation of IκBα, and NF-κB activation [18-20]. 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 other NF-κB-inducing cytokines, can drastically induce the accumulation of IKKi/ε mRNA [18-20]. 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.* [21] 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 the tumor necrosis factor receptor-associated factor. Thus, it is possible that IKKi/ε acts either 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 [3]. Most of the IκB family proteins contain a conserved DSGXXS motif, where X is any amino acid [22,23]. IKK is also able to phosphoyrlate NF-κB p65 protein at a non-consensus site, S536 [24]. The ability of IKK to exert its profound kinase activity has led to many intensive investigations exploring its possible role in other cellular events. A homology search of the gene bank protein sequence database revealed that a number of non-IκB/NF-κB family proteins also contain IKK phosphorylation 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 recently been demonstrated to be an IKK

Table 1. Confirmed and Putative IKK Substrates in Mammalian Cells

Substrates	Consensus Sites		References
Confirmed substrates:			
IκBα	31	D **S** G L D **S**	
IκBβ	18	D **S** G L G **S**	3
IκBε	17	D **S** G I E **S**	3
	70	D **S** T Y G **S**	82
p105	921	D **S** V C D **S**	
	925	D **S** G V E **T**	22
p65	535	S **S** I A D M	23
β-Catenin	32	D **S** G I H **S**	24
Putative substrates:			10
Rho-GEF	220	D **S** G L D **S**	
CDC2-related kinase 7	439	D **S** G L E **S**	
Centaurin β2	386	D **S** G N E **S**	
HNF-3α	35	N **S** G L G **S**	
Metastasis suppressor protein	321	D **S** G F I **S**	
PPARγ coactivator 1	60	D **S** G F V **S**	
TRAF6	285	D **S** G Y I **S**	
hIRS-1	423	D *g* G F I **S**	
Plakoglobin	23	D **S** G I H **S**	
Carboxypeptidase A1	175	D **T** G I H **S**	
C-Met oncogene	284	D **S** G L H **S**	
RAD51 associated protein 1	18	D **S** G N D **S**	
Chromodomain helicase	53	D **S** G S E **S**	
TNFR2	72	D **T** V C D **S**	
Tight junction protein 1 (ZO1)	810	D **S** G V E **T**	
Pannexin 2	265	D **S** G V Q **T**	
Eps8	809	D **S** G V E **S**	

substrate [10], no experimental data is yet available to suggest that IKK can phosphorylate these non-IκB/NF-κB family proteins. Recent evidence does suggest that IKKα controls keratinocyte differentiation and that IKKβ can attenuate insulin signaling as in type 2 diabetes and obesity [17,25]. Thus, in addition to NF-κB signaling, IKK appears to be involved in several other cellular signal transduction pathways by either a kinase activity-dependent or a kinase activity-independent manner.

From a pharmaceutical perspective, there are several good drugs and drug groups that might be of use for the inhibition of IKK activity. One group is the nonsteroidal anti-inflammatory drugs (NSAIDs), that include aspirin, ibuprofen and sodium salicylate. NSAIDs have been previously shown to inhibit activation of NF-κB and cytokine-induced mRNA of cell adhesion molecules [26]. Later studies reported that the effects of NSAIDs are the result of specific inhibition of ATP-binding to IKKβ [27,28], that is independent of their cyclooxygenase-2 (COX-2) inhibitory activity. A second candidate is 15d-PGJ_2, an anti-inflammatory cyclopentenone prostaglandin and a natural ligand for peroxisome proliferator-activated receptor γ (PPARγ) [29,30]. In Jurkat T cells or HeLa cells, 15d-PGJ_2 inhibits TPA- or TNFα-induced NF-κB activation in a PPARγ-independent manner [31]. A direct modification of cysteine 179 (C179) in the activation loop of IKKβ by 15d-PGJ_2 was observed in an *in vitro* IKK kinase activity assay [32]. The IC_{50} for the inhibition of IKK activity was estimated to be ~5 μM. 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-κB is desirable. However, it should be noted that the inhibitory effect of 15d-PGJ_2 on IKK and NF-κB might well be cell type or stimulus dependent. This is evidenced by the fact that 15d-PGJ_2 can potentiate LPS-induced gene expression of IL-8, an NF-κB targeting gene [33]. 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 [34-36]. The specificity and potential application of these natural products in inhibiting IKK activity however, remains to be elucidated. Finally, several relatively specific IKK inhibitors have been developed by Signal Pharmaceuticals, Millennium Pharmaceuticals and Novartis Pharma AG, respectively. Signal Pharmaceuticals developed a selective IKKβ inhibitor named SPC839 that inhibits IKKβ with nanomolar potency and IKKα with micromolar potency. It is reported that the IC_{50} of PS1145, another IKK inhibitor developed by Millennium Pharmaceuticals, on the inhibition of IKK is lower than 0.1 μM [37].

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 has been shown that MEKK1 is a far more important activator of JNK signaling than for ERK signaling [38,39]. The first piece of evidence suggesting the involvement of MEKK1 in signal-induced IKK activation was reported by Lee *et al.* [40]. In their studies, they showed that the addition of a recombinant catalytic domain of MEKK1 (MEKK1Δ) to a partially enriched fraction of nonstimulated HeLa cells stimulated an IKK-like kinase activity that phosphorylated IκBα at S32 and S36 and subsequent ubiquitination and degradation of IκBα. Follow-up studies demonstrated that overexpression of MEKK1 stimulated the NF-κB-dependent transcriptional reporter [15]. Activation of NF-κB by HTLV Tax protein was shown to require MEKK1 [41]. MEKK1 also contributes 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 can promote the proteolytic activation of MEKK1 and subsequent activation of NF-κB [42]. Further studies by Mercurio *et al.* have demonstrated the presence of a protein in the IKK complex that is recognized by an antibody raised against MEKK1 [12].

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 inactivates both kinases, whereas phosphomimetic glutamic acid substitution at these positions results in constitutively active kinases [12,14]. Nevertheless, additional studies are needed to confirm whether MEKK1 is indeed a physiological activator of IKK in cells responding to various stimuli. Indeed, a recent study by Xia *et al.* [43] 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 [44]. One study using a yeast two-hybrid screen observed an interaction between NIK and IKK, suggesting that NIK might be a direct upstream activator of IKK [45,46]. 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 [15]. When the abilities of MEKK1 and NIK to activate total IKK kinase activity are compared, most of the studies show that NIK is a more potent 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 preferentially phosphorylates 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), Fas [44], Toll-like receptors 2 and 4 [47,48], LMP1 [49] and CD3/CD28 stimulation [50]. 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 *alymphoplasia* (*aly*) contradicts this assumption. The *Alymohoplasia* mouse strain failed to develop lymphoid organs, such as lymph nodes and Peyer's patches due to a point mutation in the NIK locus [51]. The mutation of the NIK locus results in disruption of the interactions between NIK and IKKα or TRAF proteins. Further analysis indicated that the *aly* mutation does not affect TNFα-induced activation of NF-κB but only blocks lymphotoxin-mediated activation of NF-κB. Similarly, studies using cells derived from NIK-deficient mice have suggested that NIK appears to be dispensable for IKK activation induced by TNFα or IL-1 [52]. Intriguingly, lymphotoxin β induces normal NF-κB DNA-binding activity in NIK-deficient cells, whereas the same treatment fails to induce NF-κB reporter gene activity or NF-κB target gene expression [52]. 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 that activates IKK through direct phosphorylation in cells stimulated with PMA [20,53]. This novel kinase was named NAK, TANK-binding-kinase 1 (TBK1), or T2K. Pomerantz and Baltimore [20] cloned NAK using a yeast two-hybrid screen with 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 [53]. 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 [20] showed that NAK might form a ternary complex with TANK and TRAF-2, suggesting that NAK functions far more upstream of the signal cascade leading to IKK activation, whereas the *in vitro* kinase activation assay used by Tojima and co-workers [53] demonstrated that NAK was a more direct upstream kinase phosphorylating IKKβ. Interestingly, activation of endogenous NAK resultes in only S36, but not S32 phosphorylation of IκBα, a similar phenomenon as observed in recombinant IKKi/ε-mediated IκBα phosphorylation [18]. Since both IKKα and IKKβ are able to phosphorylate both S32 and S36 of the IκBα 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 [18,20,53]. 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 PI3K-dependent kinase 1/2 (PDK1/2) signaling pathways [54]. 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 [54]. However, many questions remain regarding the involvement of Akt in signal-induced IKK activation. Studies by Ozes *et al.* [55] and Xie *et al.* [56] indicated that Akt was required for TNFα- or G protein activator-induced NF-κB activation through direct phosphorylation and activation of 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 can induce 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 [55]. By contrast, Romashkova *et al.* [57] showed 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 report by Delhase and co-workers [58] showed that Akt activation induced by IGF-I fails to activate IKKα, IκBα phosphorylation and degradation, or NF-κB DNA binding in HeLa cells, the same cell line used by Ozes *et al.* [55]. Similarly, several other recent studies have showed that Akt is not involved in TNFα-induced NF-κB activation in human vascular smooth muscle cells, skin fibroblasts, or endothelial cells [59,60]. Rather, Akt might enhance the ability of the p65 (RelA) transactivation to induce transcription [61,62]. In Jurkat T-cells, Akt alone does not activate NF-κB, but is capable of potentiating NF-κB activation induced by PMA, partially by enhancing IκBβ degradation [63]. Evidence further supporting this notion comes from 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 [64]. Thus, it is possible that Akt phosphorylates IKK in a cell context- and stimulation-dependent manner. It is also quite possible that several different mechanisms are involved in Akt-regulated NF-κB activation. One question that remains 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 [65,66].

5. Mixed-Lineage Kinase 3 (MLK3)

MLK3, another member of the MAPKKK family, contains an N-terminal SH3 domain, followed by a catalytic domain and two tandem leucine/isoleucine zippers, a basic region, a Cdc42/Rac binding motif, and a proline-rich C terminus [67]. Based on these structural characteristics, MLK3 is believed to associate with a wide variety of protein modules. Studies by Hehner *et al.* [68] suggested that

MLK3 could directly associate with the IKK complex through its leucine zipper domain and phosphorylate Sl76 of IKKα and Sl77 and Sl8l 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 the consequent NF-κB-dependent transcription. MLK3 has also been shown to form a complex with a JNK scaffold protein JIP and stimulate JNK activation [67]. Thus, MLK3 may function as an integral molecule between the signaling pathways leading to the activation of NF-κB and JNK. This would provide a molecular explanation for why many stimuli induce both NF-κB and JNK simultaneously, under certain situations.

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 [69]. Transfection of 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 a TGFβ-responsive promoter [69]. However, the biochemical link between TGFβ and TAK1 has been elusive. Intriguingly, the contributions of TAK1 to signal-induced NF-κB and JNK activation have been studied intensively. Several new insights into the roles of that TAK1 plays in IKK activation have emerged in the cellular response to cytokines or Toll signals [70-75]. The first suggestion that TAK1 was 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 [76]. Other studies then demonstrated a direct physiological interaction between TAK1 and IKK in unstimulated cells [74]. 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 [71,74]. 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* signal [70]. Genetic studies and sequential chromatographic purification by Wang *et al.* [75] have shown 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 lysine 63 (K63) at each ubiquitin molecule. 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) [77]. The major questionable 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 [75,76], Ninomiya-Tsuji *et al.* [73] have shown 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 a 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 IKK, or far more distal kinases indirectly activating IKK. These kinases include Cot [50], PKCζ, PKCα [78], PKCθ [79,80], or PKR [81] *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 of this is the observation that PKCθ and Cot kinase participate in CD3-CD28 costimulation signal-induced, but not TNFα-induced, activation of NF-κB [50,80].

III. Mechanisms of Ubiquitination in NF-κB Activation

As described 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 in this process is the phosphorylation-dependent conjugation of IκB proteins with the polyubiquitin chain, a marker required for the proteasomal degradation of IκBα. Whereas the ubiquitination sites on IκBβ and IκBε have not yet been definitively identified [82-84], lysines 21 and 22 (K21 and K22) on the IκBα protein are considered as the major sites conjugated by the polyubiquitin chain [85,86].

Ubiquitin is a highly conserved and heat stable 76-amino acid protein found in virtually all types of eukaryotic cells [87]. 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-NH_2 group of a K residue of the substrate. In successive reactions, the polyubiquitin chain, is synthesized by progressive transfer of ubiquitin moieties to K48 or K63 of the previously conjugated ubiquitin molecule to forme G76-K48 or G76-K63 isopeptide bonds. An assembly factor, termed Ufd or E4, is believed to be required for this process [88]. 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 an 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 [11,89,90]. The binding of SCF to IκBα results in the association of SCF with specific E2s, including Ubc3, Ubc4, Ubc5, and Ubc9 [91,92]. These E2s are able to catalyze the ubiquitin conjugation of IκBα and the assembly of the G76-K48 polyubuquitin chain. Consistent with the likely role of SCF-β-TrCP as a ubiquitin ligase complex that conjugates the 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 that inhibits the activation of the *Drosophila* NF-κB homolog, *Dorsal* [93]. 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 the 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 have shown that this process can be antagonized by SUMO-1 (small ubiquitin-related modifier-1) modification of IκBα on the same residues where the polyubiquitin chain is conjugated [94], or by unknown product(s) of nonpathogenic *Salmonella* bacteria [95]. SUMO-1 is one of the best-characterized members of the ubiquitin-related proteins. Conjugation of SUMO-1 to substrates requires the SUMO-1-activating enzyme Aos/Uba2 as well as the SUMO-1-conjugating enzyme, Ubc9. Although substrates can be modified by SUMO-1 at several distinct sites, no multi-SUMO-1 chains are apparently formed [96]. In contrast to ubiquitination of IκBα protein, SUMO-1 conjugation does not target IκBα for proteasomal degradation [94]. The inhibition of NF-κB by certain bacterial pathogens may occur through a mechanism that affects the conjugation of SUMO-1 to IκB proteins. An example is the observation that *YopJ*, a protein product encoded by a 70-kB plasmid harbored in the *Yersinia* species that caused the Black Death plaque in the Middle Ages, inhibits MEKK1-induced NF-κB activation. Earlier studies by Orth *et al.* [97] showed that the inhibition of NF-κB by *YopJ* was through a direct interaction of *YopJ* with IKKβ but not with IKKα. Structural analysis of the *YopJ* protein by the same group later suggested that *YopJ* might be a SUMO-protease that promotes the conversion of precursor SUMO-1 to mature SUMO-1 [98]. Nevertheless, there is still no clear evidence demonstrating that *YopJ* enhances the conjugation of SUMO-1 to IκBα.

The vast majority of ubiquitination reactions in which proteins are ubiquitinated via G76-K48 assembly of the polyubiquitin chain, target protein for proteasomal degradation. Examples include the ubiquitination of IκBα, p53, cyclins, c-Jun, and others. This is, however, not the case with protein ubiquitination via G76-K63 assembly of the polyubiquitin chain. Biochemical evidence of G76-K63 assembly of the polyubiquitin chain remains elusive, but it appears to be independent of proteasomal degradation [99]. Recent studies by Wang *et al.* [75] 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 ubiquitin ligase E3 activity as evidenced 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

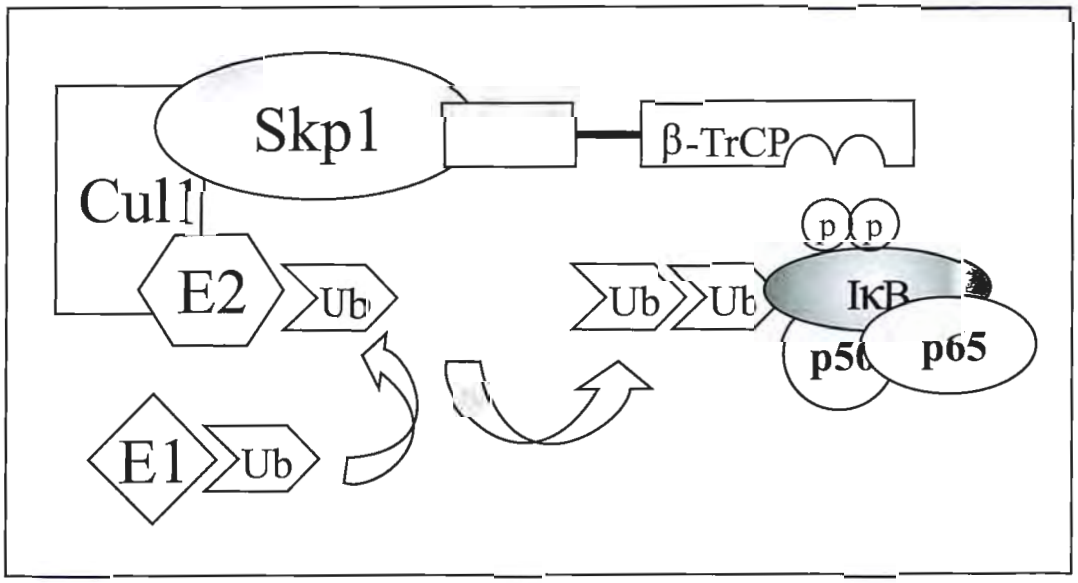

Fig. (2). SCF-β-TrCP ubiquitin ligase complex-mediated ubiquitination of IκB proteins. The basic components of this E3 complex include Skp1, Cul-1 (CDC53), and the F-box protein, β-TrCP. β-TrCP recognizes and links the phosphorylated IκB proteins to this complex allowing the ubiquitination of IκBs by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

family, and Uev1A, a ubiquitin-conjugating E2 enzyme variant [72]. In yeast and mammalian cells, both Ubc13 and Uev1A are considered the major enzymes required for the synthesis of G76-K63 polyubiquitin [100]. With chromatographic purification of HeLa cell cytoplasmic extracts, the Ubc13/Uev1A complex is found to co-eluted with TRAF6 and appears to be essential for TRAF6-induced TAK1 and subsequent IKK activation [72]. However, in *Drosophila*, the Ubc13/Uev1A-induced K63 polyubiquitination of TRAF6 is not yet been established, despite the identification of the *Drosophila* homologs of Ubc13 and Uev1A, *bendless* and *dUev1A,* respectively [9]. Interestingly, Ubc13/Uev1A was also found to interact genetically with a DNA repair protein, Rad5 [77,101], suggesting that it is 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 the pathophysiological response that results from alterations in cellular redox homoeostasis due to either over-production of reactive oxygen intermediates (ROIs) or because of a deficiency in the buffering or scavenging system for ROIs [102,103]. Typically, cells that are oxidatively stressed 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 recover 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 oxidative stress or to repair ROI-mediated damages. However, sustained oxidative stress produced during either a chronic or acute inflammatory response and/or with environmental toxicant exposure will elicit cellular toxicity.

Among all the known oxidative stress inducers, H_2O_2 and some environmental toxic metals and particles are perhaps the most potent and well studied [104,105]. Many other agents, such as TNFα, IL-1 and bacterial or viral proteins, can also induce oxidative stress [106]. Since the discovery of NF-κB, hundreds of reports have appeared suggesting that some extracellular stimuli that induce oxidative stress also activate NF-κB [107-109]. Thus, it is not surprising that many researchers have proposed a connection between ROIs and signal-induced NF-κB activation [107-109]. Some investigators have even suggested that ROIs might be the universal molecules mediating the activation of NF-κB in response to a broad range of stimuli [107]. However, the idea that ROIs directly mediate NF-κB activation has been strongly challenged [110-113]. Firstly, the temporal correlation between the generation of ROIs and NF-κB activation does not conclusively mean that ROIs are essential mediators that link upstream signals to NF-κB activation. Under certain circumstances, ROI generation may simply be a bystander signal or a secondary response evoked with NF-κB activation. Secondly, it is important to exercise caution when interpreting the inhibitory effects of different antioxidants on signal-induced NF-κB. Many antioxidants can disturb the normal cellular redox status that maintains the basal signaling potential required for activation of NF-κB or other intracellular biochemical events even under non-oxidative stress conditions. In addition, many low-molecular-weight antioxidants can inhibit NF-κB via non-antioxidant actions [114,115]. Thirdly, it should be noted that several studies have shown a failure of ROIs to activate NF-κB in different experimental systems [116,117]. Finally, there is new evidence to suggest that the DNA binding activity of NF-κB requires a reducing condition [118]. 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 [119-122].

The signal transduction pathways, such as the upstream and proximal kinases that lead to the activation of NF-κB by TNF, IL-1, Toll, LPS, and CD28, have now been clearly defined. However, only a limited amount of information is available to suggest the responsiveness of these kinases to ROIs [106,123-127]. Evidence to implicate ROIs as stimulators of IKK is based on the observed elevation of IKK activity in human epithelial cells or mouse fibroblast cells with H_2O_2 treatment [127,128]. In our own studies, we have found a modest induction of IKK activity in response to chromium(VI), a potent intracellular inducer of H_2O_2 [129]. Nevertheless, there are several contrasting reports that have appeared challenging the stimulatory effect of ROIs on IKK activity. Li and Karin [124] were unable to detect IKK kinase activity in HeLa cells stimulated with UV-C, another intracellular H_2O_2 inducer [130], despite the fact that UV-C can induce IκBα degradation and NF-κB DNA binding. Similarly, Korn *et al.* [125] reported that H_2O_2 itself fails to stimulate IKK, but rather, inhibits TNFα-induced IKK activity. It is quite likely that H_2O_2 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 or a high concentration of arsenic [32,131].

Whereas IKK activation seems to be a less favorable target 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 (132-135]. 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 [136,137]. Oxidation of the C-X-X-C motif of Trx by ROIs induces the dissociation of Trx from ASK1, thereby allowing for the dimerization of ASK1 and consequent activation of JNK. We have previously reported that

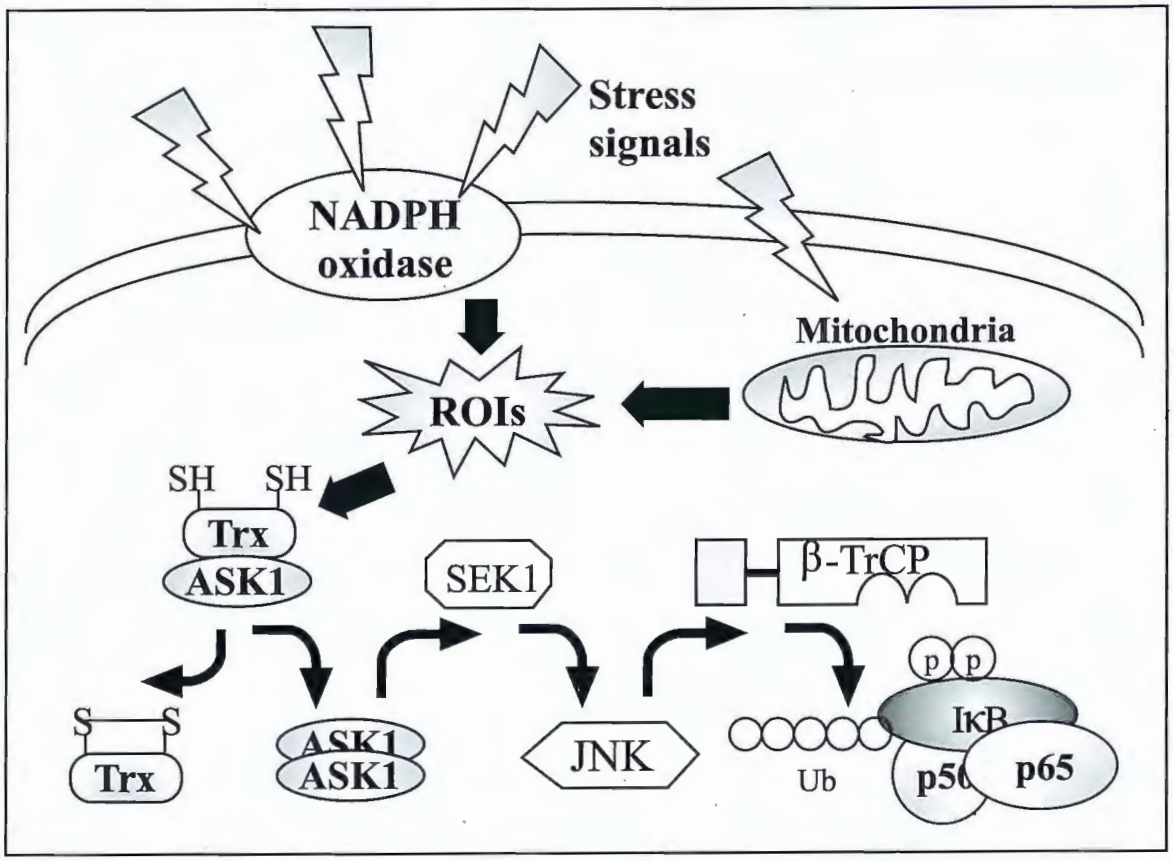

Fig. (3). Model for the ROI-induced oxidation of Trx and the subsequent ubiquitination of IκBs. Oxidation of the C-X-X-C motif of Trx induces its dissociation from ASK1, thereby allowing the dimerization and activation of ASK1. JNK is activated by SEK1 that has been phosphorylated by ASK1, leading to the accumulation of β-TrCP which is required for the processes of IκB ubiquitination.

inhibition of JNK by overexpression of a dominant negative SEK1 impairs the degradation of IκBα and the activation of NF-κB induced by vanadate [138]. This observation was further substantiated by Spiegelman *et al.* [135] who provided convincing evidence for the contribution of JNK to the signal-induced ubiquitination of IκBα protein. Activation of JNK resultes in accumulation of β-TrCP, a subunit of the SCF-β-TrCP complex that recognizes the phosphorylated DSGXXS motif within the IκBα protein and causes the subsequent ubiquitination [135]. While JNK has been implicated in the stabilization of a number of short-lived mRNAs in response to stress [139,140], it is reasonable to speculate that the JNK-mediated accumulation of β-TrCP occurs through stabilization of the β-TrCP mRNA. Indeed, analysis of the β-TrCP mRNA sequence by Spiegelman *et al.* [135] revealed a closely resembled JNK response element in addition to two AU-rich elements in the 3'-UTR region of β-TrCP mRNA. Since ubiquitination of the IκBα protein is potentially a rate-limiting step, the abundance of β-TrCP regulated by JNK may serve as an important point of regulation in ROI-induced NF-κB activation.

SUMMARY

There is growing body of evidence implicating the dysregulation of signaling pathways that lead to the activation of the NF-κB transcription factor in the pathogenesis of a variety of human diseases, including autoimmune diseases, neurodegenerative diseases, inflammation, and malignancy [2-4]. Moreover, several human diseases caused by inherited mutations of genes encoding NF-κB signaling molecules have been described [141-143]. The signal transduction pathways leading to NF-κB activation, therefore, represent attractive targets for therapeutic intervention to treat these diseases. As discussed above, tremendous advances have been made in our understanding of the upstream signaling pathways of NF-κB activation. Yet we are far from fully understanding all of the intricacies of these complex pathways. 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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