

## MINIREVIEW

# Bridging the Gap: Composition, Regulation, and Physiological Function of the I $\kappa$ B Kinase Complex

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Protein kinases that regulate the activity of specific transcription factors in response to extracellular stimuli not only are the subject of intense research but also are being chased as potential targets for development of new drugs for treatment of various human diseases. One such protein kinase is IKK, the I $\kappa$ B kinase that activates nuclear factor  $\kappa$ B (NF- $\kappa$ B) through phosphorylation of I $\kappa$ B inhibitory proteins. In this review, we summarize the discovery of IKK and recent knowledge about its composition, regulation, and physiological functions.

NF- $\kappa$ B transcription factors regulate the expression of a large number of genes that are necessary for proper functioning of the immune system and are key mediators of inflammatory responses to pathogens (2, 4, 5). NF- $\kappa$ B is also associated with cellular transformation and oncogenesis, and one of its most important, but lately discovered functions, is the activation of an antiapoptotic gene expression program (6, 29, 36, 38, 39, 42). As a transcription factor that orchestrates the inflammatory response, NF- $\kappa$ B is rapidly activated, independently of new protein synthesis, in response to signals produced during infection (e.g., bacterial endotoxins and viral double-stranded RNA) (for a review, see reference 3). NF- $\kappa$ B activation is also a transient response; this is of importance because many of the genes that are activated by NF- $\kappa$ B encode potentially toxic products such as tumor necrosis factor (TNF). The key to NF- $\kappa$ B regulation is the inhibitory  $\kappa$ B (I $\kappa$ B) proteins which retain NF- $\kappa$ B in the cytoplasm (reviewed in reference 37). In response to diverse stimuli, I $\kappa$ Bs are rapidly degraded and the freed NF- $\kappa$ B dimers translocate to the nucleus. Several years ago, it was established that the critical event which triggers the polyubiquitination and degradation of I $\kappa$ Bs via the 26S proteasome is their stimulus-dependent phosphorylation at two serine residues (residues 32 and 36 in I $\kappa$ B $\alpha$ ) that are located within their conserved N-terminal regulatory region (1, 10–12, 18, 20, 34, 40). The protein kinase that phosphorylates these regulatory sites remained elusive, and without detailed knowledge about its molecular identity, there was little progress towards a full understanding of the signaling pathways that control NF- $\kappa$ B activity. The initial hunt for such a protein kinase yielded many false candidates, such as protein kinase C, casein kinase II, and ribosomal S6 kinase (pp90<sup>rsk</sup>) (reviewed in reference 43). Although most of these kinases phosphorylate I $\kappa$ B proteins in the test tube on different serine, threonine, or

tyrosine residues, none of them was found to phosphorylate the two regulatory sites that trigger the degradation of I $\kappa$ Bs in a stimulus-dependent manner.

A large-molecular-mass (700-kDa) protein kinase activity that phosphorylates I $\kappa$ B $\alpha$  on S32 and S36 in a ubiquitin-dependent manner was also detected in extracts of nonstimulated HeLa cells (13, 25). However, this activity was not reported to be stimulus dependent, and to date, its components and molecular identity are unknown.

A careful consideration of I $\kappa$ B phosphorylation indicated that the physiological I $\kappa$ B kinase had to fulfill several criteria. Its activity should be stimulated by inducers of NF- $\kappa$ B with kinetics that are consistent with those of NF- $\kappa$ B activation, and it should phosphorylate both S32 and S36 in the N terminus of I $\kappa$ B $\alpha$  and both S19 and S23 in the N terminus of I $\kappa$ B $\beta$ . In addition, since substitution of threonines for these serines results in I $\kappa$ B mutants that are resistant to degradation, the physiological I $\kappa$ B kinase should be serine specific (18).

### IDENTIFICATION OF IKK AS THE PHYSIOLOGICAL I $\kappa$ B KINASE

To isolate a kinase that meets these requirements, DiDonato et al. (19) employed a biochemical approach to purify a 900-kDa protein kinase complex from extracts of TNF-treated HeLa cells. In preliminary experiments, this activity was found to phosphorylate I $\kappa$ B $\alpha$  at the proper serines and to discriminate against the mutant with threonine substitutions (19). Most importantly, this kinase activity was found to be rapidly stimulated by proinflammatory cytokines with the proper kinetics (19). A large-scale purification of this 900-kDa protein kinase complex, named IKK for I $\kappa$ B kinase, resulted in identification of two polypeptides, of 85 and 87 kDa, that coeluted with I $\kappa$ B kinase activity on several gel chromatography and affinity columns (19). Microsequencing and cDNA cloning identified these polypeptides as two closely related protein kinases, IKK $\alpha$  (IKK1) and IKK $\beta$  (IKK2) (19, 47). A similar approach undertaken by Mercurio and coworkers yielded identical results (31). At the same time, a two-hybrid screening conducted by Regnier et al. (32) resulted in the isolation of IKK $\alpha$  (previously identified as a protein kinase with unknown function named CHUK [16]) as a protein that interacts in yeast cells with another protein kinase called the NF- $\kappa$ B-inducing kinase (NIK). NIK is so called due to its ability to potently stimulate NF- $\kappa$ B activity in transiently transfected cells (30). Both IKK $\alpha$  and IKK $\beta$  were initially found to be cytokine-responsive I $\kappa$ B kinases whose kinetics of activation match those of I $\kappa$ B $\alpha$  phosphorylation (19, 47). In addition, expression of an antisense

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IKK $\alpha$  RNA or a kinase-defective mutant of IKK $\beta$  inhibits activation of NF- $\kappa$ B by proinflammatory cytokines, thus providing further support for the IKK complex being the long-sought-after I $\kappa$ B kinase (19, 31, 41, 47).

### COMPOSITION OF THE IKK COMPLEX

The IKK complex contains two catalytic subunits, IKK $\alpha$  and IKK $\beta$ , of 745 and 756 amino acids, respectively. In addition to a kinase domain at their N termini, IKK $\alpha$  and IKK $\beta$  contain protein interaction motifs, a leucine zipper (LZ), and a helix-loop-helix (HLH) motif at their C-terminal portions. The kinase domains are 64% identical, while the C-terminal LZ and HLH motifs exhibit 44% identity. IKK $\alpha$  and IKK $\beta$  can form homodimers and heterodimers (or tetramers) in vitro, and purified recombinant forms of each can directly phosphorylate I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  at the proper sites (26, 46).

In addition, the IKK complex contains at least one regulatory subunit, IKK $\gamma$ /NEMO, which was identified by two different and independent approaches. Using a monoclonal antibody to the IKK $\alpha$  subunit, the IKK complex was purified to near homogeneity from two human cell lines (33). This complex, which we refer to as the core complex because its isolation involved a stringent wash with 3 M urea (which may have removed loosely attached subunits), contains equimolar amounts of IKK $\alpha$  and IKK $\beta$  and two additional polypeptides, of 50 and 52 kDa (33). Microsequencing and molecular cloning revealed that these polypeptides, IKK $\gamma$ 1 and IKK $\gamma$ 2, represent differentially processed forms of the same protein (33). Complementation cloning of cDNAs whose products restore NF- $\kappa$ B activation in two cell lines that are completely defective in NF- $\kappa$ B activation resulted in isolation of the mouse homolog of IKK $\gamma$ , named NEMO (NF- $\kappa$ B essential factor) (44). Expression of IKK $\gamma$ /NEMO in these cell lines restores the ability to activate IKK and NF- $\kappa$ B in response to TNF alpha (TNF- $\alpha$ ), interleukin 1 (IL-1), double-stranded RNA, and human T-cell leukemia type 1 infection (44). These studies provided the evidence that IKK is indeed the physiological I $\kappa$ B kinase necessary for NF- $\kappa$ B activation by all of these stimuli. The requirement of IKK $\gamma$  for NF- $\kappa$ B activation was also demonstrated by an antisense RNA approach whereby cell lines made to express lower levels of IKK $\gamma$  exhibited a considerable decrease in I $\kappa$ B $\alpha$  phosphorylation, degradation, and NF- $\kappa$ B activation (33). IKK $\gamma$ /NEMO is a 419-amino-acid-long, glutamine-rich protein that lacks a known catalytic domain but contains several coiled-coil protein interaction motifs, including an LZ, next to its C terminus (33, 44). A C-terminally truncated version of IKK $\gamma$  lacking the LZ can still bind IKK $\alpha$ -IKK $\beta$  heterodimers, but once expressed, it prevents IKK activation by a number of stimuli, including TNF and IL-1 (33). Most importantly, the IKK complex assembled around the C-terminally truncated IKK $\gamma$  is refractory to activation by a variety of different stimuli (33). These results not only confirm the requirement of IKK $\gamma$  for IKK activation but also suggest a specific function whereby it connects the IKK complex to upstream activators. Most likely, these connections occur via protein-protein interactions mediated by the C-terminal LZ (Fig. 1).

Highly purified recombinant IKK $\gamma$  by itself can form up to tetramers, but it appears to bind IKK $\alpha$ -IKK $\beta$  as a dimer (33). Recombinant IKK $\gamma$  interacts directly with recombinant IKK $\beta$  but not with recombinant IKK $\alpha$  (33). Since IKK $\alpha$  and IKK $\beta$  are mostly present as heterodimers (or tetramers), it is possible that IKK $\gamma$  also interacts with IKK $\alpha$  once bound to IKK $\beta$ . A preferential interaction of IKK $\gamma$  with IKK $\beta$  would allow for differential regulation of the two catalytic subunits, such that upstream activators that interact with the C terminus of IKK $\gamma$

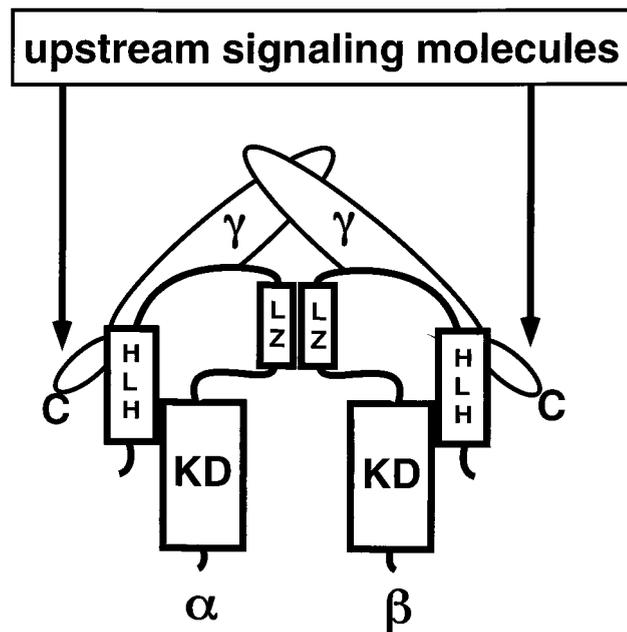


FIG. 1. Composition of the IKK core complex and its connection to upstream signaling molecules. Two IKK $\gamma$ s are associated with a heterodimer of IKK $\alpha$ -IKK $\beta$ . The interaction of IKK $\alpha$  and IKK $\beta$  is mediated through their LZs. The C terminus of IKK $\gamma$  links the IKK core to the upstream signaling molecules. KD, kinase domain.

would activate the IKK complex via its IKK $\beta$  subunit. If this is indeed the case, it is possible that IKK $\alpha$  would interact with a different regulatory subunit, similar to IKK $\gamma$ , that connects the IKK complex to a distinct set of upstream activators. Such an arrangement may allow for differential regulation of IKK $\alpha$  and IKK $\beta$  catalytic activities.

As mentioned above, recombinant IKK $\alpha$  and IKK $\beta$  form homodimers and heterodimers whose apparent molecular masses, determined by gel filtration, are 230 kDa (46). Addition of purified recombinant IKK $\gamma$  to IKK $\beta$  results in the formation of a large, 900-kDa complex (48). Interestingly, in cells lacking IKK $\gamma$ /NEMO, the IKK complex migrates as a small, 200- to 300-kDa complex and expression of IKK $\gamma$ /NEMO in these cells restores formation of the large, 900-kDa, IKK complex (44). The exact stoichiometry of the large IKK complex is yet to be determined, but these experiments suggest it may be composed solely of IKK $\alpha$ , - $\beta$ , and - $\gamma$ .

Using techniques originally developed for isolation of the c-Jun kinase (22), Cohen et al. (15) chromatographed extracts of IL-1-stimulated 293 cells on a glutathione *S*-transferase-I $\kappa$ B $\alpha$  substrate affinity column and identified an I $\kappa$ B kinase complex similar in size to IKK. In addition to IKK $\alpha$ , IKK $\beta$ , NIK, I $\kappa$ B $\alpha$ , and NF- $\kappa$ B/RelA, this complex contains a 150-kDa protein that is named IKAP (IKK complex-associated protein) (15). IKAP is suggested to function as a scaffold protein due to its ability to assemble IKK $\alpha$ , IKK $\beta$ , NIK, and NF- $\kappa$ B:I $\kappa$ B (15). It should be noted, however, that it is not clear whether the complex found by Cohen and coworkers was purified to homogeneity and whether the reported polypeptides are its only components. For instance, Cohen and coworkers did not examine whether IKK $\gamma$ , which is tightly associated with IKK $\alpha$ -IKK $\beta$ , is part of this complex. Interestingly, the interaction of IKAP with IKK $\alpha$ -IKK $\beta$  appears to be transient such that cell stimulation with IL-1 or TNF results in dissociation of the IKAP-IKK $\alpha$ -IKK $\beta$  complex (15). This may be the reason why

a polypeptide corresponding in size to IKAP was not part of the highly purified IKK complex described by Rothwarf et al., which was isolated from extracts of TNF-stimulated cells (33). It also remains to be determined whether IKAP is required for IKK activation at all and, if so, whether it is involved in responses to all NF- $\kappa$ B-activating stimuli or to only a subset of them. It is possible that while IKK $\gamma$ , which is a stoichiometric component of IKK, is required for IKK activation by all stimuli, proteins like IKAP, which are substoichiometric components, may connect IKK to a specific set of upstream activators.

### REGULATION OF IKK ACTIVITY

NF- $\kappa$ B is activated by diverse stimuli, including ligands that act through cell surface receptors (e.g., TNF and IL-1), viral RNA and specific viral transactivator proteins, and UV and gamma rays (3, 4). Recently, UV activation was shown to activate NF- $\kappa$ B through a mechanism that does not depend on either IKK activation or I $\kappa$ B N-terminal phosphorylation (8, 27), but all other NF- $\kappa$ B inducers seem to operate via IKK. Therefore the IKK complex must be able to receive and respond to all of these signals. Indeed, it is already known that the activity of IKK is stimulated by TNF- $\alpha$ , IL-1, 12-*O*-tetradecanoate-13-acetate, lipopolysaccharide, the Tax protein of human T-cell leukemia type 1, double-stranded RNA, shear stress, and ionizing radiation (9, 14, 35, 44, 45, 47). The mechanisms by which these highly diverse stimuli activate IKK are, however, poorly understood. Structure prediction programs suggest the presence of numerous docking sites for interacting proteins on IKK $\alpha$ , IKK $\beta$ , IKK $\gamma$ , and IKAP, but the search for signaling molecules that directly dock to these sites is in its infancy. Mitogen-activated protein kinase/ERK kinase kinases (MAP3Ks), such as NIK and MEK kinase 1 (MEKK1), activate IKK when overexpressed (for a review, see reference 24). NIK may potentially interact with IKK through IKAP (15). However, there is little evidence to date that NIK or MEKK1 is a physiological IKK activator. In the case of IKK $\gamma$  (but not IKAP), genetic and biochemical data clearly show that IKK $\gamma$  is essential for IKK activation in response to at least six different stimuli (33, 44), but it is not known with which upstream activators IKK $\gamma$  interacts. One would expect that at least some of the upstream activators would be found to directly interact with IKK $\gamma$ , most probably through its C-terminal LZ.

One of the central questions regarding regulation of IKK activity is, what are the specific roles of the individual subunits in its activation? IKK is activated by phosphorylation, since its treatment with protein phosphatase 2A results in its inactivation (19). Cell stimulation with TNF enhances the phosphorylation of all three IKK subunits (17). However, the bulk increase in IKK phosphorylation occurs with considerably slower kinetics than the increase in kinase activity. Phosphopeptide mapping of IKK $\beta$  (the subunit whose phosphorylation accounts for IKK activation) indicates that phosphorylation occurs at serine residues located in two regions: S177 and S181 in the T loop, and a cluster of 15 serines located between the HLH motif and the C terminus. Conversion of the T-loop serines of IKK $\beta$  to alanines prevents IKK activation, while substitution of alanine for serine in the equivalent sites in IKK $\alpha$  has no effect whatsoever on IKK activation by TNF, MEKK1, or NIK (17, 24). These experiments indicate that the IKK $\beta$  subunit is responsible for receiving the signals generated by cell stimulation with either TNF or IL-1. It is not yet clear which signals, if any, activate IKK via the IKK $\alpha$  subunit. To what extent phosphorylation of the activation sites at the T loop is due to the action of an upstream kinase and to what extent it is due to autophosphorylation are also not clear.

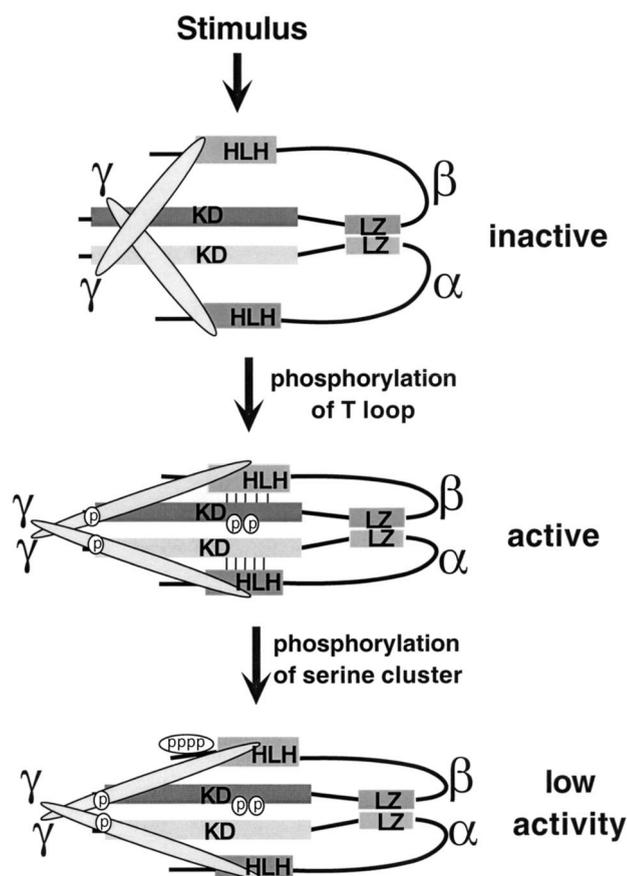


FIG. 2. Hypothetical scheme explaining the regulation of IKK by phosphorylation of the T loop and the C-terminal serine cluster of IKK $\beta$ . KD, kinase domain. See text for description of model.

Production of active recombinant IKK $\beta$  in insect cells also requires the phosphorylation of S177 and S181 within the T loop (17). Although it is possible that insect cells may contain an IKK-activating kinase, these results are more consistent with activation via autophosphorylation. It is possible that a small portion of IKK is activated initially via phosphorylation of IKK $\beta$  by an upstream kinase, which jump-starts the complex. A small number of activated IKK molecules then activate the rest by autophosphorylation.

Since prolonged NF- $\kappa$ B activation can lead to various inflammatory disorders and even death, due to excessive cytokine production, it is important not only to rapidly activate IKK and NF- $\kappa$ B in response to infection but also to quickly terminate their activities once the inflammatory stimulus has disappeared. While the T loop sites of IKK $\beta$  play a positive regulatory role, phosphorylation of the C-terminal serine cluster has a negative regulatory function (Fig. 2). Conversion of at least 10 of these serines to alanines results in a mutant form of IKK $\beta$  whose basal I $\kappa$ B kinase activity is severalfold higher than that of the wild-type form and whose activation lasts twice as long (17). Since these serines are autophosphorylated, the autophosphorylation of IKK $\beta$  appears to be an important negative autoregulatory mechanism preventing prolonged IKK activation.

A possible mechanism explaining how autophosphorylation negatively regulates IKK activity is suggested by the following findings. Mutations within the HLH motifs, which are located

immediately next to the C-terminal serine cluster, abolish the kinase activity of purified recombinant IKK $\alpha$  and IKK $\beta$  (46). These results suggest that the HLH motif is required for full IKK activation through an intramolecular interaction with the kinase domain. Indeed, coexpression of a C-terminal fragment that includes the HLH motif with a deletion mutant of IKK $\beta$  lacking the C-terminal portion restores kinase activity to wild-type levels (17). Thus, it is likely that the C-terminal portion of IKK $\beta$  (and presumably IKK $\alpha$ ), which contains the HLH motif, interacts with the kinase domain and that this interaction is required for full activity in addition to phosphorylation of the T loop. The close proximity of the C-terminal serine cluster to the HLH motif raises the possibility that its phosphorylation weakens the interaction between the HLH motif and the kinase domain, causing the kinase to reach a lower activity state (Fig. 2).

From data gathered so far, one can conclude that at least two events are required for full IKK activity. One is the interaction of the HLH motif with the kinase domain and the other is the phosphorylation of specific sites in the T loop of IKK $\beta$ . However, the order in which these two events occur and whether one depends on the other are not clear. Another mechanism that is essential for IKK function is the homo- or heterodimerization of IKK $\alpha$  and IKK $\beta$ . LZ mutations that disrupt dimerization also abolish kinase activity altogether, including autophosphorylation (46). These results may suggest that dimerization is required for transphosphorylation of the kinase domains. However, the fact that wild-type IKK $\beta$  heterodimerized with catalytically inactive IKK $\alpha$  can be fully activated (46) suggests otherwise. The exact mechanisms by which dimerization affects kinase activity is likely to be made more clear once the three-dimensional structure is solved.

Although the role of IKK $\gamma$  in transducing the activating signals to the IKK $\alpha/\beta$  is established, its mechanistic details are not clear. Since the interaction of the HLH motif with the kinase domain is essential for kinase activity, IKK $\gamma$  could stabilize this interaction. However, since purified IKK $\beta$  is very active in the absence of IKK $\gamma$  and this activity is not further enhanced by binding to IKK $\gamma$ , a structural role for IKK $\gamma$  is unlikely. As discussed above, a more likely mechanism involves the recruitment of upstream activators (kinases) that act on IKK $\beta$  through interaction with the C-terminal LZ of IKK $\gamma$ .

### GENETIC ANALYSIS OF IKK FUNCTION

The experiments discussed above strongly suggest that IKK $\beta$  is far more important than IKK $\alpha$  for activation of the IKK complex in response to proinflammatory stimuli. As a genetic test of these findings and also to elucidate the biological functions of IKK $\alpha$  (if it is not involved in proinflammatory signaling), mouse mutants that lack either IKK $\alpha$  or IKK $\beta$  were generated. The results of these gene targeting experiments confirm the results of the biochemical experiments described above: IKK $\alpha$  is not required for activation of IKK in response to proinflammatory stimuli, whereas IKK $\beta$  is absolutely essential for this response. Most interestingly, however, these experiments indicate a new role for the IKK $\alpha$  in controlling the proliferation and differentiation of epidermal keratinocytes as well as affecting (directly or indirectly) other developmental decisions, including skeletal patterning (23, 33a). IKK $\alpha$ -deficient mice are born alive but die within 30 min. The mutant mice exhibit a plethora of developmental defects, the most striking of which are their taut, thick skin, highly rudimentary limbs and tail, and shorter head. It turns out, however, that IKK $\alpha$ <sup>-/-</sup> mice do have limbs and a tail whose proximal elements are almost normal, but they are hidden under their

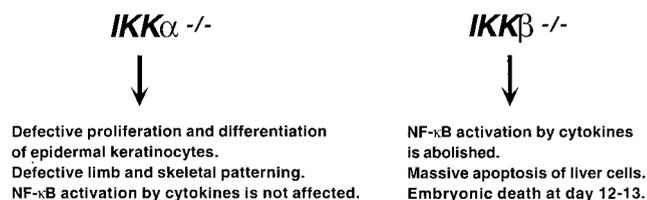


FIG. 3. Summary of defects observed in mice lacking IKK $\alpha$  or IKK $\beta$ .

thickened skin. The distal elements of the limbs are maldeveloped due to a defect in interdigital apoptosis (23, 33a).

Histochemical and microscopic examination of IKK $\alpha$ <sup>-/-</sup> skin reveals marked hyperproliferation of the epidermal layer and an almost complete absence of differentiation. Due to the absence of fully keratinized cells, the mutant skin appears to be rather sticky, which causes the limbs and tail to be "glued" to the body instead of developing as well-separated outgrowths (23). Despite these marked alterations in morphology, the activation of IKK by TNF, IL-1, or lipopolysaccharide in the fibroblasts and liver of IKK $\alpha$ <sup>-/-</sup> mice seems to be normal (23).

The loss of IKK $\beta$  results in an expected phenotype, which confirms its importance for IKK activation by TNF and other proinflammatory stimuli. IKK $\beta$ <sup>-/-</sup> mouse embryos die on day 12 to 13 of gestation due to massive liver apoptosis (28). This phenotype is essentially identical to that of p65(RelA)-deficient mice (7), except that IKK $\beta$ <sup>-/-</sup> mice die a day or two earlier. This is probably due to the more severe decrease in NF- $\kappa$ B activity in IKK $\beta$ <sup>-/-</sup> cells than in RelA<sup>-/-</sup> cells. Indeed, protein kinase and mobility shift assays indicate that IKK $\beta$ <sup>-/-</sup> cells are completely defective in activation of IKK and NF- $\kappa$ B in response to TNF or IL-1 (28a).

In summary, despite the extensive sequence similarity between IKK $\alpha$  and IKK $\beta$  and their tight association in most cell types (33, 47), these two protein kinases play different regulatory and functional roles (Fig. 3). IKK $\beta$  is essential for IKK activation by proinflammatory cytokines and for I $\kappa$ B phosphorylation. Yet, IKK $\beta$  does not have an essential role in embryonic development. The hepatic apoptosis in IKK $\beta$ <sup>-/-</sup> embryos is simply due to a defect in NF- $\kappa$ B activation, which is required for protecting the liver from TNF-induced apoptosis (21). By contrast, IKK $\alpha$  is dispensable for IKK activation or I $\kappa$ B phosphorylation in response to proinflammatory stimuli but plays an essential role in epidermal development. The signals that activate IKK $\alpha$  during keratinocyte differentiation and its relevant substrates remain to be identified.

About 3 years have passed since the initial purification of the IKK complex. We have learned quite a bit during this period, but there are still many open questions. Given the rapid pace of progress so far, it is likely that many of these questions will be answered in the near future. It is also likely that, with time, we will learn about new substrates and functions of IKK that extend well beyond the realm of NF- $\kappa$ B.

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