Minireview

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The Beginning of the End: IκB Kinase (IKK) and NF-κB Activation*

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NF-κB/Rel proteins are dimeric, sequence-specific transcription factors that control a variety of important biological decisions from formation of dorsal-ventral polarity in insects to activation of inflammatory and innate immune responses (reviewed in Ref. 1). NF-κB proteins are related through the Rel homology domain (RHD),[1] which subjects them to a particular type of regulation, centered around nuclear-cytoplasmic shuttling (reviewed in Ref. 2). The RHD serves several functions. It is the dimerization and DNA binding domain, and we have learned in atomic detail how RHDs dimerize and interact with DNA (3). In addition, the RHD contains a nuclear localization sequence (NLS), and most importantly it is the site for binding of inhibitors of NF-κB, the IκBs (reviewed in Ref. 2). The IκBs also form a small family with a core composed of six or more ankyrin repeats, an N-terminal regulatory domain, and a C-terminal domain that contains a PEST motif (reviewed in Ref. 2). By binding to NF-κB dimers, the IκBs mask their NLS and cause their cytoplasmic retention. Some IκBs, such as IκBα, contain a nuclear export sequence and when combining with NF-κB dimers in the nucleus (which the IκBs can presumably enter by diffusion) cause their exportin-mediated transport to the cytoplasm (4). Recently the three-dimensional structures of NF-κB·IκB ternary complexes (composed of the RHDs of p50 and p65 and the ankyrin repeat core of IκBα) were solved (5, 6). These fascinating structures indicate that the ankyrin repeats of IκBα form a slightly bent cylinder through a stacked arrangement of α-helices that compose their ankyrin repeats. The peptide loops that connect these helices make specific contacts with the two RHDs, whose N-terminal Ig-like repeats flank the IκB core; the C-terminal Ig-like repeats (responsible for dimerization) contact each other with the IκB cylinder lying on top of them. Although the structures solved by two independent groups differ on the way by which IκB masks the NLS located next to the C-terminal Ig-like repeats of the RHDs (5, 6) it is likely that the first two ankyrin repeats sterically hinder the binding of importins to the NLS of NF-κB.

Regulation of IκkB Turnover

Initially, NF-κB was thought to be a B cell-specific transcription factor (1). However, it was quickly recognized that NF-κB activity can be induced in most cell types upon treatment with phorbol esters, the proinflammatory cytokines, tumor necrosis factor (TNF), and interleukin 1 (IL-1) and bacterial endotoxin. Subsequently, the list of NF-κB inducers has grown to contain double-stranded (ds) RNA, viruses, and the Tax protein of HTLV-1. It was also recognized that upon cell stimulation with these inducers, NF-κB dimers translocate from the cytoplasm to the nucleus where they bind target genes and regulate their transcription. Subsequently, the nuclear translocation of NF-κB was found to parallel and depend on induced degradation of IκBs (reviewed in Refs. 2 and 7).

Potent NF-κB activators can induce almost complete degradation of IκBs (especially IκBα) within minutes. This process, which is mediated by the 26 S proteasome (8, 9), depends on phosphorylation of two conserved serines (Ser-32 and Ser-36 in IκBα) in the N-terminal regulatory domain of IκB (10–12). Homologous serines are also required for degradation of the *Drosophila* IκB homolog, Cactus (13). Even the substitution of a single serine can considerably inhibit IκB degradation. Furthermore, these serines cannot be replaced by threonine, indicating that the kinase that phosphorylates them is serine-specific (12). In the presence of proteasome inhibitors, N-terminally phosphorylated IκBα accumulates very rapidly, indicating that its phosphorylation precedes its degradation and does not result in dissociation from NF-κB (8, 9). Phosphorylated IκBs undergo without delay a second post-translational modification, polyubiquitination. The major acceptor sites for ubiquitin in IκBα are arginines 21 and 22, whose substitution with lysines considerably retards its degradation (12, 14).

Polyubiquitination involves a cascade of enzymatic reactions, the first of which is ATP-dependent and catalyzed by E1 ubiquitin-activating enzyme to form an E1-ubiquitin thioester. The second reaction is catalyzed by the E2 ubiquitin-conjugating enzymes, which receive activated ubiquitin from E1. The last step in the cascade, the transfer of activated ubiquitin from the E2-ubiquitin intermediate to the substrate, is catalyzed by a third group of enzymes, the E3 ubiquitin-protein ligases (15). The E3 group is very heterogeneous, and most of its members are poorly characterized. Recently, a cell-free system that catalyzes the ubiquitination of N-terminally phosphorylated IκBα was established and used to show that the only regulated step in the IκB degradation pathway is the phosphorylation reaction (16). By contrast, the ubiquitinating activity that specifically recognizes phosphorylated IκB is constitutive. Most importantly, Yaron *et al.* (17) have elegantly employed this cell-free system and cutting edge protein purification and sequence determination technology to molecularly identify the recognition component of the phospho-IκB-specific E3 activity. This protein, named $E3RS^{I\kappa B}$, is a member of the F-box/WD-repeat family (reviewed in Ref. 18). Interestingly, other members of this family, which contain an F-box and one or two WD or leucine-rich repeats, are essential components of E3 activities involved in regulated protein degradation (19–21). In the case of $E3RS^{I\kappa B}$, Cdc4, and Grr1, recognition of the phosphoamino acid embedded within a specific sequence is believed to be mediated by the WD repeats (17, 18). The F box, on the other hand, is responsible for binding to Skp1, which in turn binds to members of the Cullin family, such as Cdc53 (20, 21). The Cullin subunit of the E3 complex appears to be responsible for recruitment of E2-ubiquitin onto the phosphorylated substrate (18).

$E3RS^{I\kappa B}$ is identical to β-TrCP, which was previously isolated via a two-hybrid screen as a protein that binds to the phosphorylated version of the HIV protein Vpu (22). Phospho-Vpu binds CD4, a T cell membrane protein, to induce its ubiquitination and degradation. Curiously, Vpu contains a sequence very similar to the one surrounding the phosphoacceptor sites of IκBs (Fig. 1). The same sequence is also present in β-catenin, another protein whose abundance is regulated via a ubiquitin-dependent degradation pathway (23). Furthermore, genetic analysis has shown that degradation of the *Drosophila* β-catenin homolog Armadillo depends on a homolog of $E3RS^{I\kappa B}$ called Slimb (24). Thus, rather than serving as a recognition sequence for the IκB kinase, the conserved sequence surrounding the IκB N-terminal phosphoacceptor sites is a recognition site for $E3RS^{I\kappa B}$, whose binding to IκB is strictly dependent on phosphorylation of these sites (17). Indeed, the sequence similarity

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[1] The abbreviations used are: RHD, Rel homology domain; NLS, nuclear localization sequence; TNF, tumor necrosis factor; IL, interleukin; IKK, IκB kinase; LZ, leucine zipper; HLH, helix-loop-helix; IKAP, IKK complex-associated protein; IKK-K, IKK kinase.

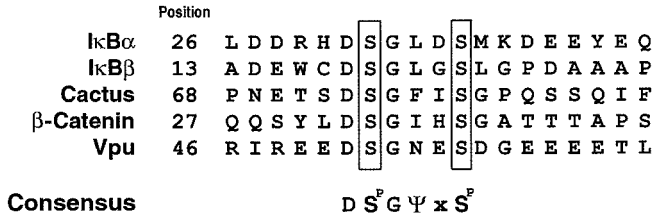

FIG. 1. **Alignment of phosphorylation sites that dictate the ubiquitin-dependent degradation of IκB proteins, β-catenin and Vpu (it is actually CD4 to which Vpu binds that is being degraded).** The consensus sequence for recognition by E3RS^{IκB}/β-TrCP is indicated. S^P, phosphoserine; Ψ, hydrophobic amino acid; *X,* any amino acid.

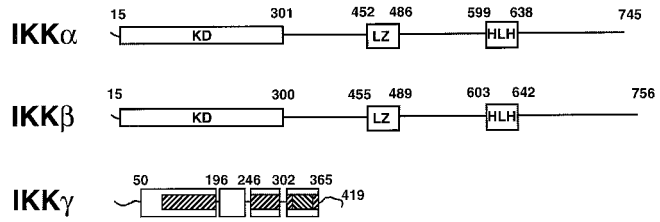

FIG. 2. **The three components of IKK.** The major structural and functional motifs of IKKα, IKKβ, and IKKγ are indicated. *KD,* kinase domain. The *open boxes* in IKKγ indicate α-helical regions within them, the *hatched boxes* denote coiled coil and leucine zipper (*LZ*) motifs.

between the IκB and β-catenin phosphorylation sites led other investigators to examine and confirm the involvement of β-TrCP in IκB ubiquitination and degradation (25). Coimmunoprecipitation experiments show that like other F box proteins, E3RS^{IκB} also associates with Skp1 and Cul1 (25). However, it remains to be established whether Cul1 rather than other Cullins is a physiological component of the E3^{IκB} complex. In addition, the particular E2 that works in conjunction with E3^{IκB} *in vivo* needs to be identified.

IκB Kinase and Its Regulation

The enzymes that catalyze the ubiquitination of phospho-IκB are constitutively active. Therefore the regulated step that dictates the fate of IκB is in most cases phosphorylation of the two N-terminal serines. As the E3^{IκB} complex may also be involved in degradation of CD4 and β-catenin, the phosphorylation step is also the one responsible for specificity in this pathway. There are only two exceptions to this universal pathway for NF-κB activation. The first is activation of NF-κB in response to UV radiation, which although dependent on IκB degradation does not involve IκB phosphorylation at the N-terminal sites (26, 27). The second exception is anoxia, which stimulates phosphorylation of IκBα at tyrosine 42 (28). The tyrosine-phosphorylated IκBα was suggested to bind to the SH2 domain of phosphatidylinositol 3-kinase, which yanks it away from NF-κB (29). Tyrosine 42, however, is not conserved in other IκBs, and therefore the universality of this pathway is questionable. The control of IκB phosphorylation in response to all other NF-κB activating stimuli rests on the shoulders of the IκB kinase (IKK) complex.

Once it became clear that the key step in NF-κB activation was IκB phosphorylation, a search for a stimulus-responsive protein kinase catalyzing this event was initiated. This effort bore fruit when a protein kinase activity that is specific for the N-terminal regulatory serines of IκBs was identified (30, 31). This activity, named IKK, is serine-specific and responsive to a number of potent NF-κB activators, most notably TNF and IL-1, which stimulate its activity with kinetics that match those of IκBα degradation (30). Furthermore, the extent to which IKK is activated seems to dictate the kinetics of IκB degradation. Gel filtration experiments suggest that IKK is a protein complex, and indeed protein purification, microsequencing, and molecular cloning resulted in identification of three IKK polypeptides. Two of these polypeptides, IKKα (IKK1) and IKKβ (IKK1), are catalytic subunits (30–33), whereas the third polypeptide, IKKγ (also known as NEMO), is regulatory (34, 35).

IKKα was also isolated through a two-hybrid screen as a protein that interacts with the mitogen-activated protein kinase kinase kinase (MAP3K), NIK (36). Although in overexpression experiments NIK acts as a potent IKK and NF-κB activator (36–39), recent experiments question its involvement in IKK activation by either TNF or IL-1 (40). Furthermore, interaction between NIK and IKKα occurs upon overexpression of the two in mammalian cells but was not detected under physiological conditions. In addition, the IKKα subunit, which was proposed to be the preferential target for NIK (41), is not directly involved in IKK activation (42).

IKKα and IKKβ have very similar primary structures (52% overall identity) with protein kinase domains at their N terminus, a leucine zipper (LZ), and a helix-loop-helix (HLH) motif at their C-terminal portion (Fig. 2). IKKγ/NEMO does not contain a recognizable catalytic domain but is composed mostly of three large α-helical regions, including a LZ (Fig. 2). Biochemical analysis indicates that the predominant form of IKK is an IKK·IKKβ heterodimer associated with either a dimer or trimer of IKKγ (34). An IKK complex-associated protein (IKAP) has also been described and proposed to be involved in IKK activation (43) but is not a readily detected constituent of the IKK complex; therefore its physiological significance and function are not clear.

Native IKK complexes purified from mammalian cells seem to be assembled from IKKα·IKKβ heterodimers plus an undetermined number of IKKγ subunits (34). Yet, cross-linking experiments indicate that *in vitro* IKKα and IKKβ can form both homo- and heterodimers in a manner that depends on integrity of their LZ motifs (33). When examined as transiently expressed proteins in mammalian cells, IKKα and IKKβ exhibit identical activation kinetics and substrate specificities (31, 32). Although highly reproducible, such experiments are misleading because the transiently expressed proteins readily interchange with their endogenous counterparts and thus are incorporated into IKK complexes (32). Thus when epitope-tagged IKKα is precipitated and its associated IκB kinase activity is measured it is not clear whether one measures its activity or those of endogenous IKKα or IKKβ with which it associates. Indeed, transiently expressed catalytically inactive IKKα and IKKβ associate with a substantial amount of cytokine-inducible IκB kinase activity (32). Nevertheless, overexpression of catalytically inactive IKKα or IKKβ inhibits NF-κB activation in response to TNF, measured by translocation of p65/RelA to the nucleus (32).

The kinase activities of IKKα and IKKβ or their abilities to be activated depend on LZ-mediated dimerization, and LZ mutations that interfere with this process abolish kinase activity (32, 33). IKKα or IKKβ activity is also abolished by mutations within the HLH motif (32, 33). These mutations, however, do not interfere with dimerization or binding to IKKγ. Rather, the HLH motif interacts with the kinase domain and can stimulate its activity when expressed in trans (42). IKK activation also requires an intact IKKγ subunit. No IKK or NF-κB activity can be elicited in IKKγ/NEMO-deficient cells that are treated with TNF, IL-1, endotoxin, or dsRNA (35). In addition, IKK complexes assembled on a mutant of IKKγ that lacks its C-terminal LZ are refractory to all of these agonists (34). These results provide a genetic proof for the importance of IKK in NF-κB activation and suggest that the C-terminal region of IKKγ is necessary for recruitment of upstream activators.

Activation of IKK depends on phosphorylation of its IKKβ subunit (42). The first evidence for the role of phosphorylation was obtained by treatment of purified, activated IKK complex with protein phosphatase 2A, which resulted in its inactivation (30). Furthermore, treatment of cells with protein phosphatase 2A inhibitor results in activation of IKK (and NF-κB). More recently, incubation of cells with TNF was shown to stimulate the phosphorylation of all three IKK subunits (42). IKKα and IKKβ are phosphorylated exclusively at serines. The location of these serines was biochemically mapped for IKKβ, and by conjecture it can be assumed that equivalent sites are phosphorylated on IKKα. Two of the IKKβ phosphoacceptors are located in its activation loop (42), a portion of the kinase domain that is involved in phosphorylation-dependent activation of other protein kinases (44). The non-phosphorylated form of the activation loop folds back onto the kinase domain and interferes with entry of ATP and peptide substrates into the catalytic pocket. Phosphorylation moves the activation loop away from the catalytic pocket, thus allowing its interaction with substrates (45). Replacement of the two phosphoacceptor serines (Ser-177 and Ser-181) of IKKβ with alanines prevents activation, whereas their replacement with phosphomimetic glutamate residues results in con-

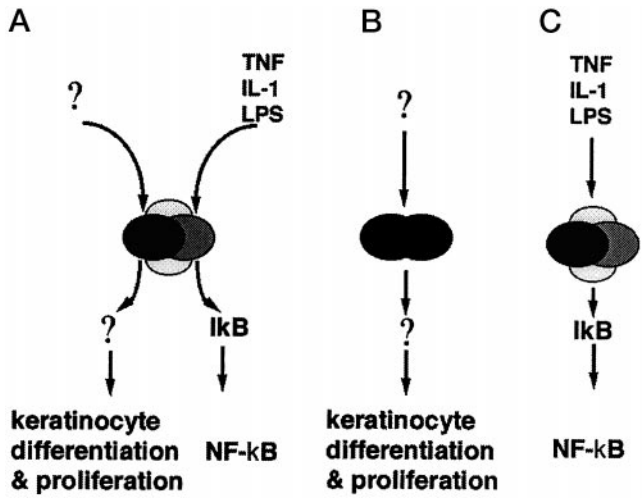

FIG. 3. **A three-step model for regulation of IKK activity by phosphorylation.** In non-stimulated cells the two catalytic subunits of IKK (IKKα and IKKβ) are associated into a heterodimer via their LZs. In this state the activation loop within the kinase domain (*KD*) is not phosphorylated, the HLH motif contacts the kinase domain, and the C-terminal phosphorylation cluster is not phosphorylated either. Upon phosphorylation of the activation loop of IKKβ and trans-autophosphorylation of the second subunit (either IKKα or IKKβ, in the case of a homodimer) IKK is activated. Once activated, in addition to phosphorylation of IκBs, IKK progressively autophosphorylates at the C-terminal serine cluster. When at least nine of the C-terminal serines are phosphorylated electrostatic repulsion alters the interaction between the activating HLH motif and the kinase domain such that the kinase reaches a low activity state. At this state IKK is more prone to inhibition by phosphatase action.

stitutive activation (31, 42). Interestingly, however, replacement of the two equivalent serines (Ser-176 and Ser-180) in IKKα abolishes autophosphorylation of this subunit but has no effect on stimulation of total IKK activity by TNF, IL-1, or the upstream kinases MEKK1 and NIK (42). These results, which underscore the biochemical differences between the two catalytic subunits, strongly suggest that IKK is activated as a result of IKKβ phosphorylation and that IKKα phosphorylation, although concurrent with that of IKKβ, is not essential for stimulation of IκB kinase activity. In other words, the IKKβ subunit and not IKKα serves as the target for upstream activators involved in proinflammatory signaling that are recruited to the complex via IKKγ.

Phosphorylation is also involved in negative regulation of IKK activity. In addition to the activation loop, IKKβ is extensively phosphorylated at its C-terminal region, which contains multiple serines (42). Phosphorylation of these sites depends on autokinase activity. Mutagenesis experiments indicate that the C-terminal autophosphorylation sites are involved in shutoff of kinase activity (42). Replacement of 9 or 10 of the C-terminal serines with alanines results in a mutant whose activation lasts four times longer than that of the wild-type enzyme, whereas substitution of the same sites with phosphomimetic glutamic acid residues results in a mutant enzyme that can hardly be activated. Based on these results, a three-state model was proposed to explain the regulation of IKK activity (42). Initially, the inactive IKK complex is not phosphorylated on its catalytic subunits. In response to upstream stimuli, IKK-Ks are activated and recruited to the complex via IKKγ. This results in phosphorylation of IKKβ and activation of IKK. We presume that initially only a small fraction of IKK is activated through direct phosphorylation by IKK-Ks. However, through intramolecular trans-autophosphorylation the activated IKKβ subunit can phosphorylate the adjacent subunit, which can be either IKKα or IKKβ (in the case of a homodimer), as well as other inactive IKK complexes through an intermolecular reaction. Indeed, the mere overexpression of IKKβ in Sf9 cells is sufficient for its activation, which depends on autophosphorylation at the activation loop. The activated IKK complexes phosphorylate the IκB subunits of NF-κB·IκB complexes, triggering their ubiquitin-dependent degradation and activation of NF-κB. Concurrently, the activated IKKβ subunits (and presumably the IKKα subunits as well) undergo C-terminal autophosphorylation. This reaction, which is unlikely to be processive, operates as a timing device such that when at least nine of the C-terminal serines are phosphorylated the enzyme reaches a low activity state. This facilitates inactivation of IKK by phosphatases once the upstream signal has disappeared. As the C-terminal autophosphorylation sites are adjacent to the HLH motif they may exert their negative effect on kinase activity by changing the conformation of this intrinsic activator domain and affecting its interaction with the kinase domain. This mode of regulation explains why IKK is usually activated in a highly transient fashion. Because of the ability of IKKβ to propagate its active state via autophosphorylation at the activation loop it is important to have an active way to reduce kinase activity and render it sensitive to inactivation by a phosphatase. Without this prolonged IKK activation would result in prolonged NF-κB activation followed by increased production of both primary and secondary inflammatory mediators. As these mediators can lead to further NF-κB activation (46), there is a genuine risk that in the absence of an efficient way to rapidly terminate both IKK and NF-κB activities even a minor proinflammatory insult would result in a major catastrophe, such as septic shock. Interestingly, constitutive IKK activation was recently detected in Hodgkin's disease cells (47). This results in constitutive NF-κB activation, which protects these cells from induction of apoptosis by radio- and chemotherapy (48). In fact, elevated NF-κB and IKK activity may protect numerous types of tumors from apoptosis-inducing therapies (49). Thus, IKK offers a reasonable target for development of new antitumor drugs.

IKK Function

The presence of two closely related, yet distinct catalytic subunits within the IKK complex is a curiosity that raises a few questions and possibilities. Are IKKα and IKKβ completely redundant and thus have identical functions? Alternatively, each subunit may be responsible for phosphorylation of different substrates and may even be subject to differential regulation. Initially, little differences between IKKα and IKKβ were observed, and it was assumed that the two have identical function. Yet more recent experiments, discussed above, suggested that IKKβ and not IKKα is involved in IKK activation by proinflammatory stimuli (42). It therefore became important to use a genetic approach to examine the relative functions of IKKα and IKKβ. This was done through the use of gene targeting technology to generate mouse strains deficient in either catalytic subunit.

The first surprising result produced by these experiments was the phenotype of IKKα-deficient mice. The complete loss of IKKα results in perinatal lethality. *IKK*$\alpha^{-/-}$ mice are born alive but die within 30 min (50). Newborn *IKK*$\alpha^{-/-}$ mice display rudimentary limbs and tail, a large omphalocele, and severe craniofacial deformity, but most striking is their skin, which is taut, shiny, and completely devoid of wrinkles. Histopathological examination reveals that the mutant mice contain limb bones of almost normal size, but they are hidden under their thickened skin (50). The major problem with the limb bones is syndactyly and absence of phalanges, whereas more proximal elements appear normal. Other notable skeletal abnormalities include a partially split sternum, fused vertebrae, and severe truncation of the skull. Yet, the most dramatic change is in the structure of the epidermis; the mutant epidermis is up to 10-fold thicker than normal, whereas the dermis appears unaltered. Increased thickness of the mutant epidermis is due to hyperproliferation of cells at the basal layer (which is normally one cell thick). In addition, there appears to be a block to keratinocyte differentiation such that instead of having a stratified epidermis the mutant mice are covered by a uniform layer of cells. The mutant epidermis lacks the upper layer of keratinized cells resulting in increased adhesiveness and stickiness. Transverse sections reveal that mutant limbs and tail are actually "glued" back to the body (50). As epidermal thickenings, such as the apical ectodermal ridge, are an important source for morphogens that control skeletal development many of the morphogenetic defects in *IKK*$\alpha^{-/-}$ mice could be secondary to a primary defect in epidermal differentiation.

The second surprise was that IKKα is not required for IKK activation by proinflammatory stimuli. Upon stimulation of IKK$\alpha^{-/-}$ embryonic fibroblasts, primary keratinocytes, or liver tissue with IL-1, TNF, or endotoxin, normal IKK activation and IκBα degradation were observed (50). Despite normal induction of IKK

activity and IκB degradation, *IKKα*$^{-/-}$ fibroblasts exhibit an approximately 50% decrease in total NF-κB DNA binding activity. Thus although IKKα does not play a primary role in IKK activation, it may still be involved in stimulating the translocation of NF-κB to the nucleus or enhancing its DNA binding activity. Regardless of the partial defect in NF-κB activation, the IKK complex in *IKKα*$^{-/-}$ cells is of normal size and exhibits normal regulation. As none of the currently available knockout mouse mutants that are deficient in any of the known NF-κB subunits or components of the IL-1 and TNF signaling pathways exhibit a similar phenotype, it is unlikely that the developmental and morphogenetic defects in *IKKα*$^{-/-}$ animals are caused by alterations in NF-κB activation. Most likely, IKKα regulates the activity of a key determinant of keratinocyte proliferation and differentiation. It is the altered regulation of this putative IKKα substrate that leads to the morphogenetic defects in *IKKα*$^{-/-}$ mice.

By contrast to IKKα, the IKKβ subunit fulfills all expectations. Although its loss also results in embryonic lethality, the phenotype of *IKKβ*$^{-/-}$ embryos is completely different from that of *IKKα*$^{-/-}$ embryos at the same developmental stage. *IKKβ*$^{-/-}$ embryos die approximately at embryonic day (E) 12.5, and histopathological examination reveals that the cause of death is massive liver apoptosis (51, 52). By comparison, *IKKα*$^{-/-}$ embryos or neonates have a perfectly normal liver. The massive increase in apoptosis of hepatocytes in *IKKβ*$^{-/-}$ embryos is strikingly similar to the major pathology observed in *RelA*$^{-/-}$ embryos, which die at E14.5 because of liver degeneration (53). Because mice that are deficient in both p65 (RelA) and p50 (NFκB1) also die at E12.5 (54), it appears that the loss of IKKβ results in a more severe decrease in NF-κB activity than the loss of p65 (RelA) alone. RelA expression is needed to protect cells from TNF-induced apoptosis (55–58). Indeed, mice that lack both RelA and TNF are viable and have normally appearing liver (59). Thus the liver degeneration in *RelA*$^{-/-}$ mice is due to increased TNF-induced hepatocyte apoptosis unopposed by NF-κB, and most likely this is the cause of death in *IKKβ*$^{-/-}$ embryos. Correspondingly, IKKβ-deficient cells are unable to activate IKK or NF-κB in response to either TNF or IL-1 (51, 52). Thus, unlike IKKα, IKKβ is absolutely required for activation of IKK and phosphorylation of IκBs.

Although these experiments provide rather definitive evidence for the different and non-overlapping functions of IKKα and IKKβ they generate a new dilemma. Does IKKα exert its morphogenetic function as a component of the "classical" IKK complex composed of IKKα, IKKβ, and IKKγ, or does it also function as a stand-alone kinase or a component of a completely different complex (Fig. 3)? An answer to this difficult question will require extensive biochemical analysis of IKK complexes in keratinocytes, the cell type in which IKKα exhibits its unique function. Once the biochemical form of IKKα involved in keratinocyte differentiation is identified, it will be possible to determine how its activity is regulated and which of its substrates plays a fate-determining role in these cells. Despite the many remaining questions, it is satisfying to witness the rapid progress in understanding IKK function and regulation, given the rather recent discovery of this important protein kinase.

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