Activation of the IκB Kinases by RIP via IKKγ/NEMO-mediated Oligomerization*

To understand the mechanism of activation of the IκB kinase (IKK) complex in the tumor necrosis factor (TNF) receptor 1 pathway, we examined the possibility that oligomerization of the IKK complex triggered by ligand-induced trimerization of the TNF receptor 1 complex is responsible for activation of the IKKs. Gel filtration analysis of the IKK complex revealed that TNFα stimulation induces a large increase in the size of this complex, suggesting oligomerization. Substitution of the C-terminal region of IKKγ, which interacts with RIP, with a truncated DR4 lacking its cytoplasmic death domain, produced a molecule that could induce IKK and NF-κB activity in cells in response to TRAIL. Enforced oligomerization of the N terminus of IKKγ or truncated IKKα or IKKβ lacking their serine-cluster domains can also induce IKK and NF-κB activation. These data suggest that IKKγ functions as a signaling adaptor between the upstream regulators such as RIP and the IKKs and that oligomerization of the IKK complex by upstream regulators is a critical step in activation of this complex.

In unstimulated cells, the transcription factor NF-κB is sequestered in the cytoplasm through interaction with inhibitory proteins known as IκBs (1). Upon stimulation by proinflammatory cytokines like TNFα, IκBs undergo phosphorylation at specific serine residues by kinases known as IκB kinases (IKKs).1 Phosphorylation marks IκBs for ubiquitination and degradation via the proteosome pathway (2–5). Degradation of IκBs allows the liberated NF-κB to translocate to the nucleus and activate the transcription of target genes (6).

The IκB kinase activity is present as a large (700–900 kDa) complex that includes two kinases designated IKKα and IKKβ (7–11) and a noncatalytic subunit termed IKKγ (also called NEMO, IKKAP1, or FIP-3) (12–15), whose function in physiologic signaling remains unclear. Genetic and biochemical studies have shown that IKKγ is essential for IKK activation by at least six different stimuli, including those generated by TNFα and interleukin-1 (12, 16, 17). Additional proteins found to interact with the IKK complex include MEK kinase (MEKK1), NF-κB-inducing kinase (NIK), receptor-interacting protein (RIP), and IKK complex-associated protein (15, 18–23). IKKα and IKKβ share significant sequence homology and contain three identical structural domains, namely a protein kinase domain at their N termini, a leucine zipper (LZ) and a helix-loop-helix (HLH) motif at their C termini. IKKγ does not contain a catalytic domain and is composed of three large α-helical regions, including a leucine zipper.

Purified recombinant IKKα and IKKβ are both able to phosphorylate IκBa and IκBβ and can form homo- and heterodimers through their LZ motifs. Mutations interfering with this dimerization abolish kinase activity (11, 24). No IKK activity can be elicited in vivo in IKKγ-deficient cells after treatment with TNFα or interleukin-1 (12, 16, 17). Moreover, IKK complexes assembled in vivo in cells expressing a truncated IKKγ lacking its C-terminal LZ were not responsive to cytokine-mediated activation (13). These results clearly highlight the importance of IKKγ in the activation of NF-κB and suggest that the C-terminal region of this protein is necessary for engagement by upstream activators. However, the mechanism by which the IKK complex is activated remains to be elucidated.

TNFα induces NF-κB activation through binding to two distinct receptors, p55 TNF-R1 and p75 TNF-R2, which are expressed in almost all cell types (25). Stimulation of the TNF receptors by TNFα promotes the assembly of two distinct signaling complexes via recruitment of different signaling proteins to the cytoplasmic tails of TNF-R1 and TNF-R2. Among these proteins TRADD, TRAF2, RIP, and FADD (26–28) are recruited by TNF-R1, whereas TRAF2, TRAF1, and TRIP (29, 30) are recruited by TNF-R2. Other proteins such as IAP1, IAP2, TANK, A20, NIK, and MEKK1 (21, 31–35) associate with the TNF receptor complexes and have also been suggested to play a role in regulation of NF-κB activation by these receptors. However, the events following assembly of the TNF-R1 or TNF-R2 complexes that lead to activation of the IKK complex need to be addressed.

Recently, NEMO/IKKγ has been shown to interact with RIP (15, 36, 37), an adapter protein that associates with the p55 receptor. RIP contains three major domains: a kinase domain at its N terminus, an intermediate domain, and a death domain at its C terminus. The use of RIP-deficient cell lines has highlighted the indispensable role of RIP in the p55 TNFα receptor-induced NF-κB activation (38, 39). Whereas RIP possesses a serine/threonine kinase domain capable of autophosphorylation, the kinase activity of RIP is not required for NF-κB activation (27, 38). Indeed, expression of a RIP mutant consist-

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1 The abbreviations used are: IKK, IκB kinase; TNF, tumor necrosis factor; TNF-R, TNF receptor; MEKK1, MEK kinase; NIK, NF-κB-inducing kinase; RIP, receptor-interacting protein; LZ, leucine zipper; HLH, helix-loop-helix; PCR, polymerase chain reaction; GFP, green fluorescent protein; SCD, serine cluster domain; ID, intermediate domain; DD, death domain.
buffer and eluted by boiling in SDS sample buffer.

**RESULTS**

**Oligomerization of the IKK Complex in Response to TNFα Stimulation**—Because TNFα is a trimer, ligation of TNF-R1 should result in a sequential oligomerization of the receptor-interacting protein RIP, followed by oligomerization of the IKK complex, which interacts with RIP through IKKα (see below and Refs. 15, 36, and 37). Therefore, if such a process occurs, this should result in an increase in the molecular mass of the IKK complex. To test this possibility, we analyzed the IKK complex elution profile and activity following chromatographic fractionation of 293T cellular extracts on a Superose-6 fast protein liquid chromatography column. We reasoned that if TNFα treatment induces oligomerization of the IKK complex, we should be able to detect a shift in the elution profile of the IKK components in extracts prepared from TNF-treated cells compared with untreated cells. Cellular extracts were prepared under mild condition to prevent dissociation of the complexes and then loaded onto a Superose-6 column. Fractions eluted from the column were analyzed for IKK activity and the presence of specific components of the IKK and TNF-R1 complexes using Western blot analysis. In extracts of unstimulated cells, the majority of IKKα and IKKγ were eluted in a peak centered around fraction 25 (relative molecular mass, ~650 kDa) (Fig. 1). In addition to this peak, a smaller amount of specific components of the IKK and TNF-R1 complexes was kept constant by inclusion of empty vector DNAs. The luciferase activity was determined with a Luciferase Assay System (Promega). A LacZ-expressing plasmid was used for normalizing transfection efficiencies.

**IKK Kinase Assay**—HeLa cells were transfected with epitope-tagged IKKα or IKKβ expression plasmids together with other indicated plasmids. 24 h after transfection, cells were lysed in a buffer containing 50 mM Tris, pH 7.5, 0.2 mM EDTA, 10 mM EGTA, 1 mM Na3VO4, 10 mM β-glycerophosphate, 5 mM NaF protease inhibitors, and 1 mM diithiothreitol. Cell lysates were immunoprecipitated with the anti-epitope monoclonal antibody conjugated on agarose beads, washed three times with the lysis buffer, and washed twice with the kinase buffer (40 mM Tris, pH 7.5, 0.2 mM EDTA, 10 mM MgCl2, 10 mM β-glycerophosphate, 200 μM Na3VO4, and 1 mM diithiothreitol). The immunoprecipitates were resuspended in 40 μl of the kinase buffer containing 5 μCi of [γ-32P]ATP (3000 Ci/mmol) and 1 μg of recombinant glutathione S-transferase-IgBo proteins as exogenous substrates of IKK. After incubation at room temperature for 30 min, the reactions were stopped by adding SDS sample buffer. The samples were analyzed by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes, followed by autoradiography.

**Confocal Microscopy**—293T cells were grown on coverslip and then transfected with the GFP-tagged (SCD) of IKKα or IKKβ together with the indicated vectors. 24 h after transfection, cells were left untreated or incubated with AP1510 for 30 min and then fixed with 4% paraformaldehyde in phosphate-buffered saline for 30 min. The coverslips were mounted on a glass slide, and the fluorescence of GFP was detected by confocal microscopy using excitation wavelength of 488 nm and detection wavelength of 522 nm. Images were Kalman-averaged.
bands. This modification could be the result of an increase in phosphorylation of IKKα and IKKγ after stimulation with TNFα and activation of the IKK complex. Indeed, biochemical analyses have indicated that the three subunits of the IKK complex undergo phosphorylation after TNF stimulation (40). Importantly, the IKK activity was associated predominantly with the fraction containing the 1.3-MDa complex, whereas little activity was detected in the 650-kDa fraction.

None of the downstream components of the TNF-R1, namely TRADD, TRAF2, and RIP, co-eluted with the activated IKK complex. Moreover, unlike IKKα or IKKγ, TNFα stimulation did not affect the distribution of TRADD, TRAF2, or RIP. These proteins were detected in fractions 27–31, which correspond to sizes of ~200 to ~500 kDa, in both unstimulated and stimulated extracts. RIP was less abundant in the fractions from the unstimulated cells. Perhaps this could be due to its association with the TNF-R1 complex in the nonsoluble membrane fraction before stimulation. Of note, prior to TNF stimulation, a small amount of the IKK components was also detected in fraction 27, thus co-eluting with TRAF2. However, TNF treatment of the cells clearly separated the elution profiles of TRADD, TRAF2, and RIP from that of the IKK components. Consequently, the molecular mass increase observed for the IKK complex following TNF treatment cannot be explained by a stable association between the IKK complex and components of the activated TNF-R1. Indeed, our results suggest that the small IKK complex (Mw ~650 kDa) is dynamically recruited to the TNF-R1, activated via oligomerization, and then released as a larger complex containing the activated kinases.

**IKKγ Mediates the Association of RIP with the IKK Complex**—Recent observations suggest that RIP recruits the IKK complex to the TNF-R1 in a stimulus-dependent manner (37). Moreover, RIP and IKKγ has been shown to interact in a yeast two-hybrid system (37). To confirm which component of the IKK complex serves as the primary target of RIP, human 293T cells were transfected with expression constructs for FLAG-tagged RIP and T7-IKKβ with or without T7-IKKγ. In the absence of RIP, IKKβ and IKKγ were not precipitated with the FLAG antibody (Fig. 2A). In the absence of ectopic IKKγ, a small amount of IKKβ was co-immunoprecipitated with RIP. Interestingly, in the presence of ectopic IKKγ, a remarkably higher amount of IKKβ co-immunoprecipitated with RIP. The ectopic T7-IKKγ was also detected in these complexes. This result shows that IKKγ mediates the interaction of RIP with the IKK complex, thus confirming the direct interaction of RIP with IKKγ.

**Enforced Oligomerization of the Central Domain of RIP Induces IKK Activation**—Based on the observation that IKKγ interacts with the intermediate domain of RIP (37), we hypothesized that following ligand-induced trimerization of the TNF-R1, the C-terminal death domain of RIP serves as a receptor docking and oligomerization domain, whereas the central or intermediate domain serves as an effector domain to recruit and activate the IKK complex via oligomerization. To test this hypothesis, we fused the intermediate domain (ID) of RIP (amino acids 286–579) to a 3-fold repeat of the FKBP12 polypeptide (Fig. 2B), which oligomerizes when it binds to the synthetic organic ligand AP1510 (41). Transient transfection of the RIP ID-FKBP12 construct into 293T cells resulted in expression of a protein of the expected size (Fig. 2D). In the absence of AP1510, only a modest NF-κB activation was observed in cells expressing the RIP ID-FKBP12 chimera (Fig. 2C). However, incubation of the RIP ID-FKBP12 transfected cells, but not the empty vector transfected cells, with AP1510 resulted in a large increase in NF-κB activity (Fig. 2C). Very little NF-κB activation was detected when the RIP ID-FKBP12 chimera was transfected with kinase-inactive IKKβ in the presence of AP1510 (Fig. 2C). AP1510-induced oligomerization also resulted in activation of IKK activity to a magnitude similar to that observed with TNFα (Fig. 2D).

To address the physiological relevance of this finding, we transiently expressed the RIP ID-FKBP12 chimera in wild type or IKKγ-deficient Rat-1 cells (12). Although NF-κB activation was detected in wild type Rat-1 cells, no activation was observed in the IKKγ-deficient 5R cells after incubation of the RIP ID-FKBP12 transfected cells with AP1510 or treatment with TNFα (Fig. 2E). This result provides genetic proof for the importance of IKKγ in RIP-induced activation of NF-κB, confirming its role as a molecular adaptor in the assembly of the RIP/IKK complexes. The inability of the RIP ID-FKBP12 chimera to induce NF-κB activation in 5R cells cannot be attributed to defects in the NF-κB pathway downstream of IKKγ.
because transfection of these cells with IKKγ can restore NF-κB activation by Tax, which is expressed stably in this cell line (Ref. 12 and data not shown). Combined, these results suggest that oligomerization of the intermediate domain of RIP is sufficient to activate the IKK complex, and this activation requires IKKγ.
FIG. 3. **NF-κB and IKK inducing activities of the truncated IKKγ-FKBP12 chimeras.** A, IKKγ interacts with IKKβ via its N terminus. 293T cells were transfected with T7-IKKβ and different FLAG-tagged full-length, N-terminally or C-terminally truncated IKKγ expression constructs. 24 h after transfection, cells were lysed, and the lysates were immunoprecipitated (IP) with anti-FLAG antibody. The immunoprecipitates were immunoblotted (IB) with anti-T7 antibody. The expression of T7-IKKβ and the different FLAG-IKKγ truncated mutants was determined by immunoblotting with anti-T7 or anti-FLAG antibodies, respectively. B, schematic representation of IKKγ-FKBP12 chimeras used in the oligomerization experiments. C, IKKγ-FKBP12 chimeras activate NF-κB in response to drug-induced oligomerization. 293T cells were transfected with 5XκB-luciferase reporter together with empty vector or the indicated IKKγ-FKBP12 expression constructs. 24 h after transfection, cells were either left untreated or incubated with either TNFα for 5 h or with AP1510 for 6 h as indicated. The luciferase activity in the transfected cell lysates was assayed and normalized as described in the legend to Fig. 1C. D, IKKγ-FKBP12 chimeras activate IKKβ in response to drug-induced oligomerization. HeLa cells were transfected with FLAG-IKKβ expression construct together with either empty vector or the indicated T7-IKKγ-FKBP12 expression constructs. 24 h after transfection, cells were treated as above, except that TNFα treatment was for 15 min and AP1510 treatment was for 30 min and then lysed. The lysates were immunoprecipitated with anti-FLAG antibody, and the IKK activity...
Enforced Oligomerization of the N-terminal Half of IKKγ Induces NF-κB Activation—Several studies have highlighted the crucial importance of IKKγ in the activation of NF-κB in response to several stimuli (12, 16, 17). In addition, the use of truncated mutants indicates that the C-terminal region of IKKγ seems to be necessary for the recruitment of this protein to upstream activators (15). Tax and RIP, two potent activators of NF-κB, have been shown to bind to a region located in the C-terminal half of IKKγ (36, 37). IKKγ is known to form a stable complex with IKKα and IKKβ in vivo (12–15, 42). To determine precisely which regions of IKKγ interact with IKKα and IKKβ, we transfected 293T cells with expression constructs encoding T7-tagged IKKα or IKKβ and several FLAG-tagged full-length or truncated IKKγ constructs. Western blot analysis of immunoprecipitates from the transfected cells revealed that full-length or C-terminally truncated IKKγ proteins that contain at least the first 200 amino acids were able to bind to IKKβ (Fig. 3A). No association was observed between IKKβ and the truncated mutants lacking the first 200 amino acids. Similar observations were obtained when the interactions were performed with IKKα (data not shown). We conclude that it is the N-terminal domain of IKKγ that interacts with IKKα and IKKβ.

Our observations suggest that IKKγ is an adaptor molecule, which links upstream regulators such as RIP to the IkB kinases (Fig. 2A). By using its C-terminal region to interact with the upstream regulators and its N-terminal region to associate with the IkB kinases, IKKγ can transmit the NF-κB activation signals from the upstream regulators to the effector kinases. One way by which the activation signals are transmitted is through oligomerization of the upstream regulators (43). Indeed, we have shown that artificially induced oligomerization of the isolated central domain of RIP, which interacts with IKKγ, is sufficient to activate NF-κB independent of the kinase and the death domains of RIP (Fig. 2C). Because the central domain of RIP interacts with the C-terminal half of IKKγ, we hypothesized that, upon TNF stimulation, RIP recruits and oligomerizes IKKγ, which in turn passes the oligomerization signal from RIP to the effector kinases, resulting in their activation. If this hypothesis is correct, then enforced oligomerization of the IKKγ N-terminal region, which links IKKγ to the IkB kinases, should activate these kinases and induce NF-κB, independent of upstream signals. To test this hypothesis, we fused full-length and several truncated IKKγ to a 3-fold repeat of the FKBP12 polypeptide (Fig. 3B). Transient transfection of these constructs into 293T cells resulted in expression of proteins of the expected size at similar levels (Fig. 3D). In the absence of AP1510, none of the transfected chimeric proteins induced significant NF-κB activation as measured by the LUC reporter assay (Fig. 3C). However, all chimeric proteins with the exception of IKKγ (1–105)-FKBP12 or IKKγ (200–419)-FKBP12, which do not interact with IKKα/β (Fig. 3A), induced

associated with FLAG-IKKβ was determined by immune complex kinase assay (KA). Expression of FLAG-IKKβ and the different T7-IKKγ truncated mutants was determined by immunoblotting with anti-FLAG or anti-T7 antibodies, respectively. E, IKKγ-(1–200)-FKBP12 induces activation of NF-κB in IKKγ-deficient cells upon drug-induced oligomerization. IKKγ-deficient (5R) cells were transfected with 5×κB-luciferase reporter together with either empty vector or IKKγ-(1–200)-FKBP12 expression construct. 24 h after transfection, cells were either left untreated or incubated with AP1510 for 6 h, and the luciferase activity was determined as in C.
a large increase in NF-κB activity after incubation of the transfected cells with AP1510 (Fig. 3C). AP1510-induced oligomerization of IKKγ-FL-FKBP12, IKKγ (1–300)-FKBP12, IKKγ (1–251)-FKBP12, and IKKγ (1–200)-FKBP12 but not IKKγ (1–105)-FKBP12 or IKKγ (200–400)-FKBP12 resulted also in activation of IκB-kinase activity (Fig. 3D). This effect was similar or superior in magnitude to that obtained after stimulation of cells with TNFα. These experiments show that the first 200 residues of IKKγ constitute the minimal sequence for activation of NF-κB. No NF-κB activation was detected when the FKBP12 constructs were co-transfected with kinase-inactive IKKβ (data not shown) nor after treatment of empty vector-transfected 293T cells with AP1510 (Fig. 3C). These observations suggest that oligomerization of the N-terminal domain of IKKγ activates the associated IKKα and IKKβ, which indicates that oligomerization of the kinases themselves may induces their kinase activity (see below).

To rule out the possibility that the IKKγ-FKBP12 chimeras function through interaction with the endogenous IKKγ protein, we examined their ability to activate NF-κB in the IKKγ-deficient 5R cells. These cells have been stably transformed with Tax, which induces activation of the IκB kinases by inter-
Oligomerization-induced Activation of the IKK Complex

Physiological Oligomerization of the IKK Complex Can Also Induce NF-κB Activation—To test the IKK oligomerization model in a more physiological context, we decided to use a physiological stimulus to induce oligomerization of the IKK complex directly without passing through the adaptor molecules TRADD, TRAP2, and RIP. To do that we linked the IKK complex directly to DR4 (TRAIL-R1), a member of the TNF-R family that can be trimmerized by its physiological ligand TRAIL (46–48) by replacing the cytoplasmic death domain (DD) of DR4 with the first 200 residues of IKKy. To test the ability of the DR4ΔDD- IKKy (1–200) chimera to activate the Ib κB kinases in response to physiological stimulation with TRAIL, we transfected it into the IKKy-deficient 5R cells and then incubated the cells with or without TRAIL. In the absence of TRAIL, no NF-κB activation was elicited after expression of the DR4ΔDD- IKKy (1–200) chimera in 5R cells (Fig. 4A). However, incubation of cells expressing the chimera, but not cells expressing IKKy (1–200) or DR4ΔDD, or cells transfected with an empty vector, with TRAIL resulted in a remarkable increase in NF-κB, as well as IKK activities (Fig. 4). These results demonstrate that physiological trimerization of the N-terminal half of IKKy, following death receptor ligation, is sufficient to fully activate the IKK complex.

IKKy Interacts with the C-terminal Serine Cluster Domain of IKKα and IKKβ—To understand how IKKy regulates the activity of the Ib κB kinases, it is important to know which region of these kinases interact with IKKy. IKKα and IKKβ have very similar primary structures with an N-terminal protein kinase domain, a central LZ domain, and a C-terminal region containing a HLH motif and a SCD (see Fig. 7a). Because the C-terminal region of IKKβ has been recently shown to play a regulatory role in the function of the enzyme (40), we decided to delete the SCD portion of this region and then determine whether the truncated kinases can still interact with IKKy. To this end, FLAG-tagged IKKy was transiently co-expressed in 293T cells together with either T7-tagged full-length or C-terminally truncated IKKα or IKKβ. Western blot analysis of immunoprecipitates from the transfected cells revealed that only the full-length but not the C-terminally truncated IKKα or IKKβ associated specifically with IKKy (Fig. 5a). Interestingly, the last 108- or 113-amino acid region of IKKα or IKKβ, respectively, which contains the SCD, was also able to interact with IKKy, suggesting that this region is sufficient for interaction of IKKy with the two kinases.

To verify this observation in a more physiological context, we constructed GFP-tagged SCDs of IKKα and IKKβ and examined them for possible dominant negative inhibitory activity. Expression of the GFP-tagged SCDs drastically inhibited the activation of NF-κB by TNFα in a dose-dependent manner (Fig. 5b). Moreover, NF-κB activation induced by enforced oligomerization of the IKKy (1–300)-FKBP12 and the RIP ID-FKBP12 chimeras was also blocked by the GFP-tagged SCDs of IKKα or IKKβ (Fig. 5b). Combined, these results show that the SCDs of IKKα and IKKβ play an important role in mediating the interaction of these kinases with IKKy and in transmitting the upstream activation signals to these kinases.

Combined with previous observations, our results suggest that RIP associates with IKKy and mediates its oligomerization. This in turn leads to oligomerization and activation of the associated IkB kinases. The use of IKKy-FKBP12 chimeras bypasses the TNF signaling pathway, because drug-induced oligomerization of the N-terminal part of IKKy results in the activation of the IKK kinases, probably via recruitment and oligomerization of their SCDs. To monitor this process in vivo, we used the GFP-tagged SCDs of IKKα and IKKβ as reporters. Transient expression of the GFP-tagged SCDs revealed a uniform cytoplasmic localization for both chimeras (Fig. 6a). Co-transfection with the IKKy (1–300)-FKBP12 chimeric protein in the absence of AP1510 did not alter this distribution (Fig. 6c). However, incubation of the cells expressing the GFP-tagged SCDs and IKKy (1–300)-FKBP12 with AP1510 resulted in a dramatic alteration of fluorescence from uniform to punctate fluorescence (Fig. 6d). The punctate fluorescence was not observed when cells expressing the GFP-tagged SCDs were treated with AP1510 in the absence of IKKy (1–300)-FKBP12 (Fig. 6b). These results represent a clear in vivo demonstration of the ability of the N-terminal part of IKKy to induce oligomerization of the Ib κB kinases through engagement of their SCDs. Finally, the punctate fluorescence was also observed when cells expressing a GFP-tagged IKKy (1–200)-FKBP12 chimera were incubated with AP1510 but not in the absence of the drug (Fig. 6, a and b), demonstrating that AP1510 indeed induces oligomerization of the IKKy-FKBP chimeras in vivo.

Enforced Oligomerization of SCD-truncated IKKα or IKKβ Induces NF-κB Activation—Following confirmation of the IKKy interaction with the SCD of IKKα and IKKβ, we proceeded to test directly whether SCD-truncated IKKα and IKKβ fused to the FKBP12 oligomerization domain can be activated by oligomerization. Different C-terminally truncated IKKα or IKKβ modules were fused to the inducible FKBP12 cassette and transfected in 293T cells (Fig. 7a). Treatment of cells expressing full-length or SCD-truncated IKKα and IKKβ fused to FKBP12 with AP1510 resulted in large increases in NF-κB activity (Fig. 7b). This, however, was not observed in cells transfected with constructs encoding HLH-SCD-truncated FKBP12 or LZ-HLH-SCD-truncated FKBP12 fusion proteins, as well as in cells transfected with a construct encoding kinase-inactive-FKBP12 fusion protein (Fig. 7b and data not shown). Consistently, immunoprecipitation of the T7-tagged FKBP12 constructs after treatment of the expressing cells with AP1510 resulted in the precipitation of a high Ib κB kinase activity for full-length and SCD truncated IKKα or IKKβ (Fig. 7c). These results confirm that direct oligomerization of IKKα or IKKβ induces their activation.

The above data also provide supporting evidence for the importance of the HLH motif in the kinase activity of both IKKα and IKKβ, because deletion of this motif prevents oligomerization-induced kinase activation. Recently, it has been shown that co-expression of an isolated C-terminal fragment containing the HLH and SCD motifs together with a C-terminally truncated IKKβ lacking this portion restores the ability to the C-terminally truncated IKKβ to be activated by TNFα (40). To test whether an isolated HLH-SCD-FKBP12 chimera can activate the C-terminally truncated IKKβ in trans after enforced oligomerization with the AP1510 drug, we transfected constructs expressing these proteins into HeLa cells and then treated the cells with TNFα or AP1510. Interestingly, both TNFα and AP1510 were able to induce the kinase activity of the truncated IKKβ in the presence but not the absence of the T7-IKKβ (HLH-SCD)-FKBP12 chimera (Fig. 7d). These observations suggest that the HLH-SCD motif can activate the kinase domain in trans after it receives the activation/oligomer-
FIG. 7. NF-κB- and IKK-inducing activities of the full-length and the SCD-truncated IKKα/β-FKBP12 chimeras. a, schematic representation of IKKα/β constructs used in these experiments. b, SCD-IKKα/β-FKBP12 chimeras activate NF-κB in response to drug-induced oligomerization. 293T cells were transfected with 5xκB-luciferase reporter together with the indicated IKKα/β-FKBP12 expression constructs. SCD constructs contain amino acids 1–640 of IKKα and amino acids 1–643 of IKKβ; Δ[HLH-SCD] constructs contain amino acids 1–592 of IKKα and amino acids 1–595 of IKKβ. 24 h after transfection, cells were either left untreated or incubated with TNFα for 5 h or AP1510 for 6 h as indicated. The luciferase activity in the transfected cell lysates was assayed and normalized as described in the legend to Fig. 2C. KI, kinase-inactive. Insets represent immunoblots of the expressed proteins. c, SCD-IKKα/β-FKBP12 chimeras phosphorylate IkBα in response to drug-induced oligomerization. HeLa cells were transfected with the indicated T7-tagged expression constructs. 24 h after transfection, cells were either left untreated or incubated with TNFα for 15 min or with AP1510 for 30 min as indicated. Cells were lysed, the lysates were immunoprecipitated (IP) with anti-T7 antibody, and the IKK activity was determined by immune complex kinase assay (KA). Expression of the different T7-IKKα/β constructs was determined by immunoblotting (IB) with anti-T7 antibody. d, the C-terminal region of IKKβ can activate in trans its kinase domain upon drug-induced oligomerization. HeLa cells were transfected with the indicated expression constructs. T7-IKKβ (HLH-SCD)-FKBP12 construct contains amino acids 559–756 of IKKβ. 24 h after transfection, cells were either left untreated or incubated with either TNFα...
Phosphorylation of the T-loop Is Required for Oligomerization-induced Activation of IKKα or IKKβ—Recent studies showed that phosphorylation of two specific serines (Ser177 and Ser181) in the T-loop of IKKβ is required for its activation. Mutation of these two serines to alanine residues prevents IKK activation by TNFα, whereas their replacement with phospho-mimetic glutamate residues causes constitutive activation of IKKβ (8, 40). Because oligomerization of the IKKs triggers their activation, it is possible that oligomerization induces phosphorylation of the T-loop serines resulting in full activation of the kinases. If this hypothesis is correct, then substitution of Ser177 and Ser181 of IKKβ or Ser176 and Ser180 of IKKα in our SCD truncated FKBP12 IKKα or IKKβ constructs with alanine residues should prevent oligomerization-induced NF-κB activation. As expected, no NF-κB activation was detected after treatment of the cells expressing the T-loop Ser to Ala mutants with AP1510 (Fig. 7e). Because kinase-inactive IKKα- or IKKβ-FKBP12 with wild type T-loop also failed to be activated following enforced oligomerization (Fig. 7b), these results strongly suggest that oligomerization-induced auto-phosphorylation of the T-loop serines causes activation of the IκB kinases.

**Discussion**

The cellular response to TNFα depends on cell type and the presence of specific signaling molecules. Engagement of the TNF receptors, TNF-R1 and TNF-R2, leads to the activation of two competing pathways: a pro-apoptotic pathway and an anti-apoptotic pathway (49). The anti-apoptotic pathway, which involves the activation of NF-κB via the IKK complex, is known to be initiated by recruitment of adaptor and effector molecules like TRADD, RIP, and TRAF proteins on the trimerized TNF receptors. Very recently RIP has been shown to bind to IKKy in a stimulus-dependent manner (37). However, its is not yet clear how interaction of RIP with IKKy could result in activation of the IKK complex.

A potential model that could explain how the IKK complex is activated after TNF-R1 ligation is activation by oligomerization. In support of this model, we have shown that following TNFα stimulation, the IKK complex undergoes a structural change that leads to a significant increase in its molecular mass, as observed using gel filtration. Interestingly, only the highest molecular weight complexes possessed IKK activity. This molecular mass increase could not be attributed to a stimulus-dependent, stable association of the IKK complex with the TNF receptor components, because these eluted in later fractions. Moreover, although a small amount of the IKK complex co-eluted with the activated TNF-R1 components in some fractions, no IKK activity was associated with these fractions. A previous study also reported that the activity of the IKK kinases found in the TNF-R1 complex after immunoprecipitation with a TNF-R1-specific antibody was significantly less than that found in the IKK complex after direct immunoprecipitation with an IKKy specific antibody after TNF stimulation (37), suggesting that the released and oligomerized IKK complex is the active form of IKK. Thus, prior to TNF stimulation, the IKK components exist as a ~650-kDa heteromeric complex signal from an upstream regulator such as TNFα. However, the requirement for TNFα can be bypassed if the HLH-SCD motif is linked physically to the FKBP12 oligomerization motif. In this case, the activation/oligomerization signal can be relayed directly to the kinase domain by AP1510 independent of the upstream regulators. Moreover, the activation signal must pass through the SCD motif to activate the kinase domain. This is because in the absence of the SCD, the remaining portion of IKKα or IKKβ cannot be activated by upstream signals such as those triggered by TNFα (Fig. 7c, right panel). However, AP1510 can activate the SCD-truncated kinases if they are physically linked to the FKBP oligomerization motif (Fig. 7, b and c).

**Fig. 8. A model of TNFα-induced activation of the IKK complex via oligomerization of RIP and IKKγ.** See text for details. KD, kinase domain.
complex of IKKγ, IKKB, and IKKa. Following TNF stimulation, TNF-R1-bound RIP recruits this IKK complex through interaction with IKKγ and induces its oligomerization. This oligomerization results in activation of the IκB kinases. The activated, high molecular mass ~1.3 MDa IKK complex is then released from the TNF receptor and is able to phosphorylate the IκB proteins.

Because IKKγ has been shown to interact with the intermediate domain of RIP (37), it is therefore conceivable that the death domain of RIP functions as a receptor docking domain and an oligomerizer of the intermediate domain, which in turn could induce clustering-dependent activation of the associated IKK complex upon receptor trimerization. This hypothesis was supported by the finding that enforced oligomerization of the intermediate domain of RIP can activate the IKK complex and NF-κB. Moreover, using rat embryonic fibroblasts lacking IKKγ, we demonstrated that IKKγ is essential for NF-κB activation by the oligomerized RIP intermediate domain. These results suggest that TNFα signal transduction involves oligomerization of the intermediate domain of RIP, which promotes activation of the proximal IKK complex, and that IKKγ is required to transduce this activation signal.

The C-terminal half of IKKγ interacts with the intermediate NF-κB effector domain of RIP, whereas its N-terminal half interacts with IKKα and IKKB. Tax, another known activator of the IκB kinases, has also been shown to activate the IKK complex by interacting with the C-terminal part of IKKγ (36). Based on these observations, the N-terminal half of IKKγ constitutes the effector domain of this molecule, whereas the C-terminal domain is likely to function as an oligomerization domain whose major role, after connection to upstream activator like RIP, is to induce clustering-dependent activation of IKKs (Fig. 8). This hypothesis was tested using several C-terminally truncated IKKγ fused to a 3-fold repeat of the oligomerization cassette FKBP12. The first 200 N-terminal residues constituted the minimal sequence required for IKK and NF-κB activation following oligomerization with AP1510. Thus, clustering of the N-terminal effector domain of IKKγ is sufficient for activation of the downstream IKKs.

Because AP1510 may induce extensive oligomerization of the IKK complex, which might be taken as a nonphysiological stimulus, it was therefore important to validate our observations, to examine the effect of a more “physiological” oligomerization of IKKγ. For this purpose, we constructed a DR4ΔDD-IKKγ (1–200) chimera, where the cytoplasmic DD of DR4 (TRAIL-R1) was replaced with the first 200 residues of IKKγ, and tested its ability to induce IKK and NF-κB activation following TRAIL stimulation. Like TNFα, the biologically active form of TRAIL is a homotrimer as suggested by its crystal structure (46). It is therefore reasonable to assume that trimerization of DR4 by its ligand constitutes a physiological transmembrane signaling. Interestingly, ligation of the DR4ΔDD-IKKγ (1–200) chimera induced NF-κB as well as IKK activation in the IKKγ-deficient 5R cells (Fig. 4). Importantly, this activation was comparable or superior in magnitude to the one obtained using the IKKγ-FKBP12 chimeras. This result shows that regulated clustering of the N-terminal effector domain of IKKγ is sufficient for activation of the downstream IKKs and that the upstream activation signal must be relayed to the IKKs through the N terminus of IKKγ.

Previous studies showed that IKKγ is associated with IKKα and IKKB in unstimulated cells (12–15, 42). Our data show that IKKα and IKKB interact with IKKγ via their SCDs. Because IKKγ may oligomerize by binding to RIP, this interaction induces activation of the kinases perhaps via oligomerization.

Interestingly, direct oligomerization of either IKKα or IKKB by SCD deletion mutants fused to the inducible FKBP12 oligomerization cassette activated both kinases. Based on this we postulate that the function of IKKγ is to transmit the upstream oligomerization signal to the IKKs through its interaction with their SCDs. Given that IKKγ is physically associated with the SCD of IKKα, any stimulus that could induce oligomerization of IKKγ would also lead to oligomerization of the IKKs themselves. Consistent with this, we found that the SCD of IKKα or IKKB are potent dominant negative inhibitors of TNFα- and RIP-induced NF-κB activation, perhaps because of their ability to block the sites of interactions between IKKγ and the IKKs. Moreover, SCD-truncated IKKα or IKKB were unable to be activated following TNF stimulation, demonstrating that the SCD plays an essential role in transmitting the activation signal to the kinases.

Activation of the IKK complex depends on phosphorylation of IKKα/β, because treatment of the purified activated IKK complex with the protein phosphatase 2A results in its inactivation (9). Phospho-peptide mapping of IKKB revealed that phosphorylation occurs at serines 177 and 181 located in the T-loop and in the SCD (40). In the case of IKKB, conversion of the two phosphoacceptors Ser177 and Ser181 to alanines prevents its activation, whereas conversion to phospho-mimetic glutamate residues causes its constitutive activation (40), suggesting that phosphorylation of these serines is essential for activation. However, it is not known whether this phosphorylation is due to the action of an upstream kinase or to autophosphorylation. Three kinases, NIK, MEKK1, and Akt (19–22, 50, 51), have been proposed to regulates NF-κB activation. However, very recent observations raise doubts about their involvement in the physiological regulation of IKK activation (40, 52, 53). Because direct oligomerization of the SCD-truncated IKKs in the absence of any other stimuli induces activation of these kinases, we believe that oligomerization triggers autophosphorylation of the T-loop serines. This process could be explained by the proximity model of activation. IKKs have low basal kinase activity in the unphosphorylated ~650-kDa IKK complex. Oligomerization could change the conformation of IKKs bringing the T-loop serines of one precursor molecule in close proximity to the kinase active site of an adjacent molecule resulting in autophosphorylation of the T-loop in trans (Fig. 8). Once the T-loop serines are phosphorylated, the IKKs become fully active. In support of this model, we demonstrated that mutations of the T-loop serines to alanine inhibit oligomerization-induced activation of the IKKs. Furthermore, overexpression of native IKKα or IKKB in baculovirus-infected Sf9 cells has been shown to lead to their activation in the absence of extracellular stimuli, and this activity was abolished when mutations preventing LZ-mediated dimerization were introduced (11, 24). This result can be explained by postulating that overexpression of IKKα or IKKB results in their oligomerization and autophosphorylation, thereby mimicking IKKγ-induced oligomerization after cytokine treatment. In our case, direct oligomerization of the IKK kinases bypasses the initial steps in TNFα signaling, resulting in IKK autophosphorylation, activation, and induction of NF-κB.

Although we have provided strong evidence that oligomerization of the IKK complex plays an important role in its activation, we cannot ignore the possibility that other mechanisms of activation might also be important. Direct phosphorylation of IKKα/β by other kinases such NIK, MEKK1, or Akt could also activate the IKK complex, providing a second level of regulation to this important survival pathway.

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REFERENCES

1. Baldwin, A. S., Jr. (1996) *Annu. Rev. Immunol*. 14, 649–683
2. Brown, K., Gerber, S., Carlson, L., Franzoso, G., and Siebenlist, U. (1995) *Science* 267, 1485–1488
3. Burke, J. R., Miller, K. R., Wood, M. K., and Meyers, C. A. (1998) *J. Biol. Chem.* 273, 12041–12046
4. Scherer, D. C., Brockman, J. A., Chen, Z., Maniatis, T., and Ballard, D. W. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 11259–11263
5. Chen, Z., Hagler, J., Palombella, V. J., Melandri, F., Scherer, D., Ballard, D., and Maniatis, T. (1995) *Genes Dev.* 9, 1586–1597
6. Wirth, T., and Baltimore, D. (1998) *EMBO J.* 7, 3109–3113
7. Begnini, C. H., Song, H. Y., Gao, X., Goeddel, D. V., Cao, Z., and Rothe, M. (1997) *Cell* 90, 373–383
8. Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J., Young, D. B., Barbosa, M., Mann, M., Manning, A., and Rao, A. (1997) *Science* 278, 860–866
9. DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. (1997) *Nature* 388, 548–554
10. Wornier, J. D., Cao, X., Cao, Z., Rothe, M., and Goeddel, D. V. (1997) *Science* 278, 866–869
11. Zandi, E., Rothwarf, D. M., Delhase, M., Hayakawa, M., and Karin, M. (1997) *Cell* 91, 243–252
12. Yamazaki, S., Courtois, G., Bessia, C., Whiteside, S. T., Weil, R., Agou, F., Kirk, H. E., Kay, R. J., and Israel, A. (1998) *Cell* 93, 1231–1240
13. Rothwarf, D. M., Zandi, E., Naluli, G., and Karin, M. (1998) *Nature* 395, 297–300
14. Mercurio, F., Murray, B. W., Shevchenko, A., Bennett, B. L., Young, D. B., Li, J. W., Pascual, G., Mitiwala, A., Zhu, H., Mann, M., and Manning, A. M. (1999) *Mol. Cell. Biol.* 19, 1526–1538
15. Li, Y., Kang, J., Friedman, J., Tarassishin, L., Ye, J., Kovalenko, A., Wallach, D., and Horwitz, M. S. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 1042–1047
16. Harbaj, E. V., Good, L., Xiao, G., Chiluk, M., Ciojir, M. E., Rivera-Walsh, L., and Sun, S. C. (2000) *Oncogene* 19, 1448–1456
17. Rudolph, D., Yeh, W. C., Wakeham, A., Rudolph, B., Nallainathan, D., Potter, J., Elia, A. J., and Mak, T. W. (2000) *Genes Dev.* 14, 854–862
18. O'Connell, M. P., Ultsch, M. H., Hurst, A., Totpal, K., Spencer, D. M., Wandless, T. J., Schreiber, S. L., and Crabtree, G. R. (1993) *Science* 262, 1019–1024
19. Li, Q., Lu, Q., Hwang, J. Y., Buscher, D., Lee, K. F., Izipisa-Belmonte, J. C., and Verma, I. M. (1999) *Genes Dev.* 13, 1322–1328
20. Yang, X., Chang, H. Y., and Baltimore, D. (1999) *Mol. Cell.* 4, 319–325
21. Chu, Z. L., Shin, Y. A., Yang, J. M., DiDonato, J. A., and Ballard, D. W. (1999) *J. Biol. Chem.* 274, 15297–15300
22. Jin, D. Y., Giordano, V., Kibler, K. V., Nakano, H., and Jeang, K. T. (1999) *J. Biol. Chem.* 274, 17402–17405
23. Yang, X., Chang, H. Y., and Baltimore, D. (1999) *Mol. Cell.* 3, 253–261
24. Cha, S. Y., Kim, S. M., Choi, Y. H., Sung, B. J., Shin, N. K., Shin, H. C., Sung, Y. C., and Oh, B. H. (1999) *Immunol. Cell Biol.* 77, 633–640
25. Pan, G., O'Rourke, K., Chinnaiyan, A. M., Gentz, R., Ebner, R., Ni, J., and Dixit, V. M. (1997) *Science* 276, 111–113
26. Hymowitz, S. G., O'Connell, M. P., Uitsch, M. H., Hurst, A., Topol, K., Ashkenazi, A., de Vos, A. M., and Kelley, R. F. (2000) *Biochemistry* 39, 633–640
27. Barkett, M., and Gilmore, T. D. (1999) *Genes Dev.* 13, 1029–1033
28. Ozes, O. N., Mayo, L. D., Gustin, J. A., Pfeffer, S. R., Pfeffer, L. M., and Donner, D. B. (1999) *Nature* 401, 82–85
29. Romashkova, J. A., and Makarov, S. S. (1999) *Nature* 401, 86–90
30. Israel, A. (2000) *Trends Cell Biol.* 10, 129–133
31. Pujiit, T., Ware, M., Widmann, C., Oyer, R., Russell, D., Chan, E., Zaitsev, V., Clarke, J., Tyler, K., Oka, Y., Fanger, G. R., Henson, P., and Johnson, G. L. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 7272–7277
Activation of the IκB Kinases by RIP via IKKγ/NEMO-mediated Oligomerization
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