Roles for Homotypic Interactions and Transautophosphorylation in IκB Kinase (IKKβ) Activation*

The nuclear factor-κB (NF-κB)/Rel family of transcription factors participates in a wide range of biological activities including inflammation, immunity, and apoptosis. NF-κB is kept inactive in the cytoplasm in unstimulated cells by virtue of the masking of its nuclear localization sequence by bound IκB protein. Cellular stimuli trigger the destruction of IκB proteins and the liberation of NF-κB to enter the nucleus and activate gene expression. A multisubunit IκB kinase complex (IKK) phosphorylates IκB proteins and mediates the activation of NF-κB by promoting the destruction of IκB proteases. We find that an intact leucine zipper in IKKβ proteins triggers their polyubiquitination and their subsequent recognition and degradation by the proteasome. The IKK complex contains two catalytic subunits, IKKα and IKKβ, and a noncatalytic subunit, NF-κB essential moddier (IKKε). IKK activation depends upon the phosphorylation of residues in the activation loop of IKKβ and the subsequent activation of IKKβ kinase activity. However, the events contributing to IKKβ phosphorylation are not well understood. Here, we present evidence that the activation of IKKβ depends on its ability to form homotypic interactions and to transautophosphorylate. We find that an intact leucine zipper in IKKβ is necessary for homotypic interactions, kinase activation, and phosphorylation on its activation loop. Enforced oligomerization of an IKKβ mutant defective in forming homotypic interactions restores kinase activation. Homotypic interactions allow IKKβ molecules to transautophosphorylate one another on their activation loops. Finally, the oligomerization of IKKβ is stimulated by tumor necrosis factor α in cultured cells. Our findings support a model whereby ligand-induced homotypic interactions between IKKβ molecules result in IKKβ phosphorylation and consequently IKK activation.

The IκB kinase complex (IKK) functions as a mediator of NF-κB activation in response to multiple stimuli by phosphorylating IκB inhibitor proteins and causing their degradation (1, 2). The IKK complex contains three distinct subunits, namely IKKα, IKKβ, and NEMO (or IKKγ) (1, 2). Subunit reconstitution experiments in yeast and mammalian cells suggest that IKK is composed of a NEMO homodimer bound together with either an IKKα/IKKβ heterodimer or an IKKβ homodimer (3). Several lines of evidence have indicated that IKKα and IKKβ have distinct biochemical functions. Gene disruption experiments in mice have demonstrated that IKKβ plays an essential role in NF-κB activation in response to most stimuli, whereas IKKα seems to be largely dispensable (1). Also, IKKβ and IKKα have been shown to have different substrate preferences in vitro. IKKβ has been shown to have a higher specific activity for phosphorylating IκBα, whereas IKKα has a preference for using the NF-κB2 (p100) precursor as a substrate (4).

TNF-α and interleukin-1 stimulate the phosphorylation of IKKβ on two serine residues, Ser179 and Ser181, found in its activation loop. Phosphorylation on both of these sites is required for IKK activation (5). However, the regulatory mechanisms governing this phosphorylation event are not understood. It has been proposed that the activation of IKK depends upon its recruitment to the TNF-α receptor signaling complex upon ligand binding (6, 7). Although it seems that only a small fraction of total IKK is recruited to the membrane, activation of this fraction of IKK may be sufficient to stimulate the activation of other remaining inactive IKK complexes through transautophosphorylation events.

One possible mechanism whereby IKK may initially be activated is through the activity of an IKKβ kinase. Although many candidate IKKβ kinases (based on overexpression transient transfection studies) have been proposed in the literature, only a few of these kinases have been shown genetically in cultured cells or animal models to be important for IKK activation. Two of these IKKβ kinases, MEKK3, and ζPKC, have been implicated (based upon gene disruption experiments) to be upstream regulators of IKK (8, 9). MEKK3 was shown to be required for IKK activation in response to TNF-α and interleukin-1 signaling in mouse embryonic fibroblasts (8). In contrast, ζPKC was found to be important in mice for IKK activation in response to TNF-α in the mouse lung but not in mouse embryonic fibroblasts (9). Recently, TAK1 was found to be important for IKK activation in HeLa cells in experiments using small interfering RNAs (10). It seems likely that different cell types may rely on distinct IKKβ kinases to activate IKK.

Another possible mechanism whereby the initial pool of IKK may be activated is through an induced proximity mechanism. In such a scenario, the oligomerization of an IKKβ activator and interactor such as receptor interacting protein may induce the proximity of IKK complexes and facilitate mutual transautophosphorylation (11, 12). Of note, we and others have shown...
that enforced oligomerization of IKK subunits including IKKβ by artificial chemical agents can lead to enhanced IKK and NF-κB activity (11, 12). Although it has also been shown that the overexpression of active IKKβ leads to its autophosphorylation, the ability of IKKβ molecules to transautophosphorylate series on its activation loop has not been yet demonstrated. Furthermore, the oligomerization of any IKK subunits in vitro in response to stimuli has also not been shown.

In this report we provide evidence that IKKβ activation requires the ability of IKKβ molecules to oligomerize and to phosphorylate one another on their activation loops. An intact leucine zipper in IKKβ is required for homotypic interactions, kinase activation, and activation loop phosphorylation. Furthermore, enforced oligomerization of an IKKβ mutant defective in making homotypic interactions restores kinase activation. Homotypic interactions permit IKKβ molecules to transphosphorylate one another in vivo on their activation loops. Last of all, TNF-α stimulates the homotypic interactions between IKKβ proteins in cultured cells. Our results suggest a model whereby ligand-induced IKKβ homotypic interactions lead to IKKβ transautophosphorylation and consequently IKKβ activation.

EXPERIMENTAL PROCEDURES

Cell Culture—Human embryonic kidney 293T cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum. All cells were grown in a 37°C incubator with 5% CO2. Transfections were performed with LipofectAMINE (Invitrogen), according to the manufacturer’s instructions.

Plasmids—To create wild-type and mutant FLAG-tagged and HA-tagged IKKβ expression constructs, standard PCR procedures were followed using pcDNA3-FLAG-IKK and pcDNA3-IKK as vectors. Wild-type and mutant HA-IKKβ plasmids (gifts of J. DiDonato) were used as PCR templates. pcDNA3-FLAG-IKKβ/M472S-(1–54) as a substrate to measure the activity of IKKβ proteins.

Reactions were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane and probed with antibody for 2 h at 4°C with protein G-Sepharose, and immuno precipitates were washed three times with IP lysis buffer. All immunoprecipitates were boiled in SDS-PAGE sample buffer and resolved on a 7.5% poly acrylamide gel. Proteins were transferred to polyvinylidene difluoride membrane and probed with FLAG M2, phospho-IKKβ antibody (Cell Signaling), or anti-FLAG antibody (Cell Signaling). To minimize the detection of the IgG heavy chain, protein A conjugated to horseradish peroxidase (Zymed Laboratories Inc.) was used as a secondary antibody for anti-NEMO immunoblots.

For coimmunoprecipitation assays, cells were lysed in EBC150 (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 0.1 mM Na3VO4, 1 mM dithiothreitol) supplemented with a mixture of protease inhibitors (Roche Applied Science). Lysates were incubated with antibody and protein G-Sepharose, and immunoprecipitates were washed three times with IP lysis buffer. All immunoprecipitates were boiled in SDS-PAGE sample buffer and resolved on a 7.5% polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride membrane and probed with FLAG M2, phospho-IKKβ (Cell Signaling), or phospho-IκBα antibody (Cell Signaling). To minimize the detection of the IgG heavy chain, protein A conjugated to horseradish peroxidase (Zymed Laboratories Inc.) was used as a secondary antibody for anti-NEMO immunoblots.

For coimmunoprecipitation assays, cells were lysed in EBC150 (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 0.1 mM Na3VO4, 1 mM dithiothreitol) supplemented with a mixture of protease inhibitors (Roche Applied Science), and lysates were incubated with the appropriate antibody for 2 h at 4°C and with protein G-Sepharose for an additional 1 h. All immunoprecipitates were boiled in SDS-PAGE sample buffer and resolved on a 7.5% polyacrylamide gel.

Kinase Assays—To measure IKKβ activity, cells were lysed in kinase lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 30 mM NaF, 2 mM sodium pyrophosphate, 0.1 mM Na3VO4, 1 mM dithiothreitol) 24 h after transfection, and anti-NEMO immuno precipitates were incubated in 20-μl reactions containing 50 mM Tris, pH 7.5, 10 mM MgCl2, 15 μM ATP, 1 mM dithiothreitol, 0.5 μg of GST-LE-Ba(1–54), and 5 μCi of 35P-ATP (ICN) at 30°C for 20 min. Reactions were terminated by the addition of SDS-PAGE sample buffer and resolved on a 10% polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride membrane and exposed to x-ray film. GST-LeBa(1–54) was purified from DH5α transformed with pGEX-LeBa(1–54) (a gift of M. Karin). For oligomerization-inducing experiments, transfected 293T cells were treated with 100 nM AP1510 or vehicle (ethanol) for 30 min prior to cell lysis.
RESULTS AND DISCUSSION

It has been observed previously that recombinant IKKβ purified from mammalian or insect cells is an active kinase (13–17). This activity has been attributed to phosphorylation of Ser179/Ser181 in the activation loop because a purified IKKβ mutant with alanine substitutions at Ser179/Ser181 is inactive when purified under the same conditions (5, 13). This result could be attributed to the endogenous activity of an IKKβ kinase, autoactivation by IKKβ molecules, or a combination of the two. To study the mechanism whereby IKKβ autoactivation may proceed, we first sought to construct a version of IKKβ that would not autoactivate. To achieve this goal, we chose to disrupt the leucine zipper motif in IKKβ. Previous reports have suggested that the leucine zipper of IKKα is important for homotypic interactions with itself and heterotypic interactions with IKKβ (15). However, the leucine zipper of IKKβ has yet to be characterized functionally. To generate a mutant of IKKβ with a nonfunctional leucine zipper, we substituted the methionine residue at position 472 within the predicted leucine zipper motif of IKKβ with a serine residue (M472S). This mutation rendered IKKβ defective in binding to wild-type IKKβ but not NEMO in communoprecipitation experiments (Fig. 1A and data not shown). Furthermore, this mutation significantly impaired the kinase activity of IKKβ. The M472S mutant demonstrated significantly reduced autophosphorylation activity and kinase activity toward recombinant IκBα (Fig. 1B). These results suggest that autoactivation by IKKβ in cells requires homotypic IKKβ interactions.

To determine whether the M472S mutation affected the ability of IKKβ to be phosphorylated on its activation loop, we immunoprecipitated epitope-tagged wild-type and mutant IKKβ proteins from transfected cells and performed immunoblotting using an antibody that specifically recognizes Ser181 of IKKβ. We compared wild-type IKKβ with the M472S mutant and a mutant of IKKβ with both Ser179 and Ser181 mutated to Ala (S179A/S181A). We found that in contrast to wild-type IKKβ, both the M472S and S179A/S181A mutants were not detectably phosphorylated on Ser181 (Fig. 2). Thus oligomerization of IKKβ seems to be required for the phosphorylation of residues in its activation loop, thereby leading to kinase activation. If the impaired kinase activity of the M472S mutant was caused by impaired phosphorylation of the activation loop, mimicking the phosphorylation of these residues in this mutant should be able to restore kinase activity. To mimic phosphorylation of IKKβ, we mutated the two serines in the activation loop of the M472S mutant to glutamic acid residues (M472S/S179E/S181E). Similar glutamic acid substitutions alone have been shown to yield a constitutively active kinase (17). We found that introduction of the glutamic acid substitutions restored the kinase activity of the M472S mutant, as
assessed by in vitro kinase assays (Fig. 3A). We wanted to see whether similar results were also seen with IKKβ kinase activity in vivo as well. Expression of the M472S mutant was able to elicit IkBα phosphorylation in vivo only when glutamic acid substitutions were made in the activation loop (Fig. 3B). Thus, the M472S mutant is inactive because of its inability to stimulate phosphorylation in its activation loop.

Given that the M472S mutant is impaired both in oligomerization and kinase activity, a likely possibility is that oligomerization contributes to kinase activation. If the inactivity of the M472S mutant was caused primarily by the inability to oligomerize, enforced oligomerization of the M472S mutant should lead to its activation. To test this idea, we fused the M472S mutant to three tandem repeated oligomerization domains of Fpk (Fpk3). This protein tag can be induced to oligomerize by the cell-permeable artificial ligand AP1510 (18). We compared the kinase activity of Fpk3 fusion protein in the presence of AP1510 or vehicle treatment. For comparison, we also fused versions of the M472S mutant containing activation loop substitutions to alanine or glutamic acid residues. In the presence of the oligomerizer AP1510, the kinase activity of the M472S mutant was restored with respect to both IkBα phosphorylation and autophosphorylation (Fig. 4). M472S mutants also containing activation loop substitutions to alanine or glutamic acid residues were inactive and constitutively active, respectively. Also, their activities were unaffected in the presence of oligomerizer, suggesting that the activating effect of AP1510 proceeded through phosphorylation of Ser179 and Ser181 (Fig. 4). Thus, IKKβ oligomerization can result in IKKβ phosphorylation and activation.

One can imagine that the oligomerization of IKKβ might lead directly to IKKβ activation through transautophosphorylation or indirectly through an IKKβ-associated kinase. To examine whether transphosphorylation of IKKβ molecules contributed to phosphorylation of the activation loop, we cotransfected the dead kinase K44M mutant along with different amounts of a constitutively active IKKβ/S179E/S181E mutant. Of note, the K44M mutant is not phosphorylated on Ser181 and cannot autophosphorylate itself (Fig. 1B and data not shown). After transfections, we immunoprecipitated the K44M mutant from cells and performed immunoblot analysis with phospho-IKKα antibody. We found that phosphorylation of Ser181 in the K44M mutant was induced by IKKβ/S179E/S181E. In contrast, a version of the K44M mutant carrying an M472S substitution as well could not be phosphorylated (Fig. 5). Thus, IKKβ transphosphorylation contributes to IKKβ activation loop phosphorylation.

To examine whether oligomerization of IKKβ occurred in response to TNF-α, we cotransfected two IKKβ constructs with different epitope tags and performed coimmunoprecipitation assays after cell stimulation with TNF-α for various times. To perform this experiment, we had to use low levels of expression plasmids, as higher expression of IKKβ led to constitutive oligomerization and activation. We found that TNF-α stimulation of cells resulted in the coimmunoprecipitation of IKKβ proteins (Fig. 6). These data provide evidence that IKKβ oligomerization can be induced by a physiological stimulus in vivo.

In this report, we provide evidence for a model of IKK activation whereby ligand-induced IKKβ homotypic interactions lead to transautophosphorylation and consequently IKK activation. Although our results indicate that enforced oligomerization of an IKKβ mutant defective in forming homotypic interactions is sufficient to restore activation, it is unclear from...
our experiments whether oligomerization of IKKβ alone is sufficient to activate IKKβ. A model in which IKKβ molecules autoactivate one another necessarily implies that an "initial pool" of active IKKβ exists. One could imagine that this pool of active IKKβ molecules might be derived from a preexisting fraction of IKKβ in unstimulated cells. Alternatively, this pool of IKKβ may be the product of the activity of an inducible kinase such as MEKK3, PKC, or TAK1 (8–10). The biochemical mechanism underlying the phosphorylation of IKKβ is likely to be complicated further by other events such as the phosphorylation and ubiquitination of NEMO (5, 19–22). Another unresolved issue is whether oligomerization and phosphorylation of IKKβ are separable or interrelated events. For instance, in addition to amplifying the pool of active IKKβ molecules through transautophosphorylation, IKKβ oligomerization may conceivably facilitate the recognition of inactive IKKβ molecules by an IKKβ kinase. Current investigations are focused on clarifying these and other important issues regarding the mechanism of IKK activation.

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