The mechanistic relationship of phosphorylation of the C terminus of IKKB with phosphorylation of its T-loop kinase domain within the IKK complex remained unclear. We investigated the regulatory role of the serine cluster residing immediately adjacent to the HLH domain and of the serines in the NEMO/IKKγ-binding domain (NBBD) in the C-terminal portion of IKKB in MEFs deficient in IKKB and IKKα and in yeast reconstitution system. We show that phosphorylation events at the C terminus of IKKB can be divided into autophosphorylation of the serine cluster adjacent to the HLH domain and phosphorylation of the NBBD/YBD. Autophosphorylation of the serine cluster occurs immediately after IKK activation and requires IKKγ. In MEFs, this autophosphorylation does not have the down-regulatory function on the IKK complex that was previously described (1). On the other hand, phosphorylation of the NBBD/YBD regulates IKKγ-dependent phosphorylation of the T-loop activation domain in IKKB and, hence, IKK complex activation. Our study suggests that, within the IKK complex, modulation of the NBBD/YBD by IKKγ is upstream to the T-loop phosphorylation.

Phosphorylation of inhibitors of κB (IκB) is catalyzed by the 700–900-kDa enzyme complex IκB kinase (IKK) (2–4). IKK is composed of two homologous catalytic subunits IKKα and IKKβ (85 and 87 kDa, respectively), and an unrelated regulatory 52-kDa subunit IκKγ (5) also known as NEMO (NF-κB essential modulator) (6). IKKγ is required for the stimulation of IKK by upstream signals such as TNF, Tax, lipopolysaccharide, and interleukin 1 (5, 6).

IκBs regulate the activity of the NF-κB transcription factors. NF-κB dimers control the transcription of hundreds of genes involved in immunity, stress, and regulation of apoptosis and cellular proliferation. In resting cells, most NF-κB dimers are bound to inhibitory IκB proteins and sequestered in the cytoplasm (7). Diverse stimuli, including cytokines, bacterial and viral products, oxidants, and mitogens lead to activation of IKK, which phosphorylates two regulatory serine residues on IκBs (8). This phosphorylation leads to recognition by the protein, β-TrCP, and to polyubiquitination by a specific ubiquitin ligase (9, 10). After ubiquitination, the IκB proteins are rapidly degraded by the proteasome.

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2 A 2001 PEW Scholar in Biomedical Sciences. To whom correspondence should be addressed: USC/Norris Comprehensive Cancer Center, 1441 Eastlake Ave., Norris 6429, Los Angeles, CA 90033. Tel.: 323-865-0644; Fax: 323-865-0645; E-mail: Zandi@usc.edu.

3 The abbreviations used are: IκB, inhibitors of κB; IKK, IκB kinase; TNF, tumor necrosis factor; IL, interleukin; GST, glutathione S-transferase; HA, hemagglutinin; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; HLH, helix-loop-helix; MEF, mouse embryonic fibroblasts; TRAF, TNF receptor-associated factor.
to alanines prevents IKK activation in mammalian cell lines, whereas conversion of these residues to glutamic acids makes the kinase constitutively active, indicating that the kinase is activated by phosphorylation of the T-loop (1, 3). Phosphorylation of the T-loop of IKKβ, which occurs when IKK is activated, is followed by progressive phosphorylation of the cluster of serines at its C terminus (1). This phosphorylation was suggested to play a role in down-regulating the activity of IKK in HeLa cells (1). A mutation mimicking phosphorylation in the NEMO/γ binding domain (NBD/γBD) of IKKβ diminishes its ability to activate NF-κB, suggesting that phosphorylation of this residue, in particular, may have regulatory significance (33).

In this study, we investigated the regulatory role of the serine cluster residing immediately after the HLH domain and the serines in the NBD/BD through IKK activation, and requires IKKγ. In MEFs, this autophosphorylation does not have the previously reported down-regulatory function (1). On the other hand, phosphorylation of the NBD/γBD regulates IKKγ-dependent phosphorylation of the T-loop activation domain in IKKβ and, hence, IKK complex activation. Our study puts regulation of the NBD/γBD through IKKγ upstream of the T-loop phosphorylation and IKK activation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Establishing Stable Pools of MEFs**—Mouse embryonic fibroblasts (MEFs) deficient in IKKβ (34) or IKKα and IKKβ (35) were grown in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum with antibiotics and maintained at 37 °C, 5% CO2. To establish stable pools of HA-tagged wild type and C-terminal serine to alanine mutants of IKKβ, corresponding plasmids (pRc-B-actin), (1) carrying a neomycin resistance gene were transfected using Lipofectamine Plus™ (Invitrogen). Stable pools of MEFs for each construct were selected by growing cells in 2 mg/ml G418 sulfate (Cellgro, Mediat). Mutants of IKKβ residing immediately after the HLH domain and the serines in the NBD/BD through IKK activation, and requires IKKγ. In MEFs, this autophosphorylation does not have the previously reported down-regulatory function (1). On the other hand, phosphorylation of the NBD/γBD regulates IKKγ-dependent phosphorylation of the T-loop activation domain in IKKβ and, hence, IKK complex activation.

**Kinase Assays and Immunoblots**—GST-IκBα-(1–54) was used as a substrate for IKKβ because it contains the regulatory serines but lacks other residues that could be phosphorylated nonspecifically. It was expressed in bacteria and purified using glutathione-Sepharose beads (Amersham Biosciences).

Various peptides containing wild-type or mutant versions of the C terminus of IKKβ were also produced so that they could be tested as possible substrates for IKKβ. Some of these peptides contain mutations in which serines were mutated to alanines, and all of these substrates spanned from the helix-loop-helix through the C terminus of IKKβ. DNA was amplified by polymerase chain reaction with full-length IKKβ as template using Pfu Turbo (Stratagene, La Jolla, CA), digested, and inserted into the bacterial expression vector pET-HET-N1, which contains a Myc tag at the 5’-end and a His6 tag at the 3’-end of the cloning site. DNA sequences of the constructs were verified by automated sequencing (Microchemical Core Facility/USC Norris Cancer Center). Plasmids were transformed into the bacterial strain BL21 and induced with isopropyl-1-thio-β-D-galactopyranoside. The bacterial pellet from 500-ml culture was suspended in 50 ml of phosphate-buffered saline containing 6 mM guanidine hydrochloride, sonicated for 3 min, frozen and thawed twice, and brought to a final concentration of 1% Triton X-100 and 5 mM β-mercaptoethanol. The lysate was cleared by centrifugation at 15,000 rpm for 30 min at 4 °C and incubated with 1 ml of pre-equilibrated Ni-NTA superflow agarose (Qiagen) for 1 h at 4 °C. Unbound proteins were washed away, and the bound proteins were renatured with a series of 50-ml buffers containing 4 M, 2 M, 1 M, 0.5 M, and 0 M urea in phosphate-buffered saline with 10 mM imidazole, 1% Triton X-100, and 5 mM β-mercaptoethanol. The IKKβ peptides were eluted with 200 mM imidazole in 1-ml fractions, and the positive fractions were pooled and dialyzed in phosphate-buffered saline with 1 mM dithiothreitol and 10% glycerol.

For most kinase assays, purified human IKKβ from SP9 cells (12), or gel filtration-purified or immunoprecipitated IKK complex from yeast was incubated for 30 min at 30 °C in a 30-μl reaction mixture containing 20 mM Tris, pH 7.6, 20 mM MgCl2, 20 μM cold ATP, 2 mM dithiothreitol, [γ-32P]ATP (ICN), and 2 μg of protein substrate. The reactions were terminated by the addition of SDS-PAGE sample buffer and heated for 5 min at 97 °C. Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (Bio-Rad), and labeling was detected by PhosphorImager (Molecular Dynamics).

For some experiments, IKK activity was tested using a range of IκBα concentrations (from 4–183 μg/ml). The protocol was the same as above only the substrate concentration was varied, and the incubation time was shortened to 10 min.

**Cloning and Expression of IKKs in Yeast**—The plasmid pESC-trp-met-HA-IKKβ containing HA-IKKβ under the methionine promoter was described previously (11). To co-express similar levels of IKKγ and IKKβ, HA-IKKγ and its methionine promoter were inserted into the pESC-trp-met-HA-IKKβ plasmid. Various point mutations were generated in IKKβ by PCR using Pfu polymerase (Stratagene). These PCR products were digested and recloned into the vectors pESC-trp-met-HA-IKKβ and pESC-trp-met-HA-IKKβ met-HA-IKKγ. The mutated regions were verified by sequencing. Plasmids were transformed into Saccharomyces cerevisiae strain YPH 499 (Stratagene) using lithium acetate as described (Stratagene pESC Yeast Epitope Tagging Vectors instruction manual).

2 ml of overnight cultures of yeast were grown in selective drop-out medium (Q-Biogene) containing 4 mM methionine (Q-Biogene) to suppress IKK expression and then expanded into 400 ml of selective non-inducing medium. The yeast were grown at 30 °C with shaking at 300 rpm before transfer into inducing medium (without methionine) and induced overnight (at 30 °C with shaking). For harvesting and lysing the yeast, all steps were performed at 4 °C unless otherwise indicated. They were first washed in 400 mM (NH4)2SO4, 200 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 10% glycerol, 2 mM p-nitrophenyl phosphate containing protease inhibitors (2.5 μg/ml leupeptin, 20 μg/ml aprotinin, 2.5 μg/ml antipain, 2 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 0.1 μg/ml chymostatin, and 1.1 μg/ml phosphoramidon). The pellets were resuspended in an equal volume of lysis buffer (20 mM Tris, pH 7.6, 20 mM NaF, 20 mM β-glycerophosphate, 0.5 mM Na2VO4, 2.5 mM sodium metabisulfite, 5 mM benzamidine, 1 mM EDTA, 0.5 mM EGTA, 10% glycerol, 300 mM NaCl, 1% Triton X-100, 2 mM dithiothreitol, 2 mM p-nitrophenyl phosphate with protease inhibitors), transferred to yeast protein extraction vials (Q-Biogene), and frozen at −80 °C. To lyse the yeast, the vials were thawed on ice, disrupted using a Q-Biogene Fast-Prep apparatus for 20 s on speed 6, and vortexed for 5 min. The vials were spun at 10,000 × g, the supernatant was collected, and the pellets were resuspended in 0.67 ml of lysis buffer. The extraction procedure
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was repeated an additional three times. The combined supernatants were clarified by centrifugation for 3 min at 3000 × g followed by ultra-centrifugation for 1.5 h at 65,000 × g.

HA-IKK complex was then purified by gel filtration. 0.5 ml of extract (2–5 mg total protein) was loaded onto a Superose 6 gel filtration column (Amersham Biosciences) and samples were fractionated at a flow rate of 0.3 ml per minute. The gel filtration buffer contained 20 mM Tris, pH 7.6, 20 mM NaF, 20 mM β-glycerophosphate, 0.5 mM Na2VO4, 2.5 mM sodium metabisulfite, 5 mM benzamidine, 1 mM EDTA, 0.5 mM EGTA, 10% glycerol, 300 mM NaCl, and 0.1% Brij 35.

Alkaline Phosphatase Treatment—Approximately 6 μg of yeast extract were immunoprecipitated using 1 μl of anti-HA antibody (USC/Norris core facility) in radioimmunoprecipitation assay buffer (20 mM Tris, pH 8.0, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM PNPP) followed by binding to protein G-Sepharose beads (Amersham Biosciences). Immune complexes were pelleted and washed once with radioimmunoprecipitation assay buffer and once with CIP buffer (calf intestinal phosphate buffer from New England Biolabs containing 0.1 mg/ml bovine serum albumin, 2.5 μg/ml leupeptin, 20 μg/ml aprotonin, 2.5 μg/ml antipain, 2 μg/ml pepstatin, 0.1 μg/ml chymostatin, and 1.1 μg/ml phosphoramidon). Pelleted proteins were then incubated for 15 min at 37°C in a 30-μl reaction containing 10 units of calf intestinal phosphatase in CIP buffer. The alkaline phosphatase treatment was stopped by addition of SDS-PAGE sample buffer and heated for 5 min at 95°C. For untreated controls, the addition of calf intestinal phosphatase was omitted.

In Vivo Labeling—3.5-ml cultures of yeast were grown in selective medium containing 4 μg methionine to suppress IKK expression until they reached an optical density of 0.7–1.0. Then the yeast were pelleted and resuspended in 1 ml of selective medium without methionine (to induce IKK expression) and containing 1.4 mM of [32P]orthophosphate. The yeast were radiolabeled and induced for 5 h, then washed with yeast wash buffer containing protease and phosphatase inhibitors, resuspended in 500 μl of yeast lysis buffer containing protease and phosphatase inhibitors, and frozen at −80°C. The yeast were lysed as described above, and the extract was clarified by microcentrifugation for 1 min at 10,000 × g followed by 20 min at 18,000 × g.

To remove contaminating nonspecific immune complexes, the extracts were first immunoprecipitated using a nonspecific FLAG antibody (Sigma) and complexed to protein G-agarose (Amersham Biosciences). Then, HA-IKK was immunoprecipitated using monoclonal antibodies directed against HA (USC/Norris core facility) followed by binding to protein G-agarose. The beads were washed repeatedly in radioimmunoprecipitation assay buffer (20 mM Tris, pH 8.0, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM PNPP) before elution for 5 min at 97°C in SDS-PAGE loading buffer. The samples were electrophoresed using SDS-PAGE and transferred to polyvinylidene difluoride (Bio-Rad). Radiolabeling was detected by PhosphorImager.

Cyanogen Bromide (CNBr) Digestion—Yeast cells were 32P-labeled and IKK was isolated as described above. For cyanogen bromide digestion, the immunoprecipitated samples were electrophoresed on an 8% SDS-PAGE gel. Autoradiography was used to locate the protein bands which were cut from the gel. The acrylamide slices were transferred to a siliconized tube, crushed with a pipette, and resuspended in 200 μl of 70% formic acid. Cyanogen bromide was added to a final concentration of 17 mg/ml, the samples were overlaid with argon gas, and digestion was allowed to proceed for 17 h at room temperature in the dark. Samples were microcentrifuged for 5 min, and the supernatants were transferred to siliconized tubes. The acrylamide was rinsed three times with 250 μl of ddH2O, and the rinses were combined with the digested sample. The samples were dried overnight. The peptides were electrophoresed on a 16.5%T 3% C Tris Tricine gel and visualized using a PhosphorImager.

RESULTS

Mutating C-terminal Serine Residues to Alanine in IKKβ Does Not Alter Down-regulation of IKK Complex in MEFs—At least two regions of IKKβ are phosphorylated on serine residues in response to stimulation. Phosphorylation of T-loop serines (177 and 181) in the kinase domain of IKKβ are essential for IKK activation, and phosphorylation of at least ten serines (region spanning amino acid 664 to its end) in its C terminus was shown to play a role in down-regulation of IKK in HeLa cells in the background of endogenous IKKβ. However, because a given IKK complex could contain 6 to 9 catalytic subunits (11), expression of mutated forms of IKKα or IKKβ in mammalian cells could result in the formation of heterocomplexes containing endogenous wild-type along with mutated forms. For example, when kinase-defective mutants HA-IKKαK6 or HA-IKKβK6 were expressed in HeLa or HEK 293 cells, immunoprecipitation of the mutant kinases resulted in precipitation of very active and TNF-inducible IKK complex (4). This clearly indicates that mutant IKKα or IKKβ were incorporated into the endogenous IKK complexes. Considering this, changes seen in the kinetics of IKK regulation may have been due to the formation of heterocomplexes, when the activity of the C-terminal serine to alanine mutants of IKKβ were tested in HeLa cells in the background of endogenous IKKβ (1). Here we examined the activities of the same mutant IKKβs used in Delhase and co-workers (34), in embryonic fibroblasts derived from IKKβ-deficient mouse (MEFβ−/−). We generated stable pools of MEFβ−/− cells expressing either wild-type IKKβ, or M10, L3SA, or M12 mutants. In the M10 construct, 10 serines C-terminal to the helix-loop-helix are mutated to alanines, whereas the last 4 serines remained unchanged, and in the M12 construct, these same 10 serines as well as Ser733, Ser740, and Ser750 are mutated to alanines. IKKβL3SA has three serines (Ser733, Ser740, and Ser750) mutated to alanines. The activities of IKK complexes were determined by the immune kinase complex assay in these cells before and after treatment with TNFα for 5, 15, 30, 60, and 120 min. As shown in Fig. 1A, there is no obvious difference between the activation and down-regulation kinetics of IKKβ wild type and IKKβM10. The same is true for the IKKβL3SA and IKKβM12 mutants (Fig. 1A). In all cases, the reconstituted IKK wild type or mutants show very little activity in untreated cells and reach peak activity at 15 min, similar to native IKK as previously reported (4). The activity of wild type or mutated IKKβ is down-regulated at 30 min and remains low up to 120 min (Fig. 1A). The only major difference in IKK activity is that for the M12 and L3SA mutants (which contain mutations in the serines in the yBD where IKK has faster activation kinetics (Fig. 1A). This occurs independently of the mutations of the 10 preceding serines. The L3SA and M12 mutants show reasonably strong TNF-induced activity at 5 min (Fig. 1A). These experiments were repeated several times and similar results were obtained (data not shown). Results in Fig. 1A indicate that the ten serines preceding the NBD/yBD of IKKβ do not play a role in down-regulating the activity of IKK complex. On the other hand, serines 733, 740, and 750 within and around the γ-binding domain may play a role for IKK regulation (see below).
We further examined in vitro whether there is a difference in activities between wild-type IKKβ and IKKβM10 in the absence of IKKα. IKK complexes containing IKKγ with either wild-type IKKβ or IKKβM10 were expressed in yeast and partially purified by gel filtration. The activities were compared over a range of concentrations of IκBα substrate (from 4 to 183 μg/ml, Fig. 1B). The activity of the IKKβM10 was similar to the activity of wild-type IKKβ over the whole range of substrate concentrations. This indicates that rendering the serines at the C terminus of IKKβ unphosphorylatable neither increases nor decreases kinase activity toward IκBα. For this kinase assay, there was a 10 min. incubation of enzyme in the presence of substrates, and the level of IKKβ seemed to be significantly lower at the end of the assay for samples incubated with low concentrations of IκBα (compared with the level of IKKβ after incubation in high concentrations of IκBα). This indicates that somehow, the higher concentrations of substrate stabilized the enzyme during the assay. Because of this, it was not possible to quantitatively compare the kinetic parameters of the wild-type IKKβ to IKKβM10 containing complexes. Nevertheless, qualitatively, both complexes show very similar activities.

The C Terminus of IKKβ Is Autophosphorylated in Yeast in an IKKγ-dependent Manner—Previous work (1) suggested that the C terminus of IKKβ becomes phosphorylated following IKK activation via autophosphorylation in mammalian cells. Here we used the IKK reconstituted in yeast to examine IKKγ-dependence of the autophosphorylation of the C terminus of IKKβ. A routine Western blot of IKKs expressed in yeast using HA antibodies revealed a pronounced band shift of IKKβ when co-expressed with IKKγ (Fig. 2A). This marked band shift induced by the presence of IKKγ occurred for wild-type IKKβ and IKKβ γBD AA (a mutant of IKK in which serines 740 and 750 are changed to alanine, see also Fig. 3A) but was not observed for the M10 and M12 mutants. This suggests that the serine residues C-terminal to the helix-loop-helix are phosphorylated. The greatly reduced band shift for M10 and M12, in which most of the C-terminal serines are rendered incapable of phosphorylation, suggests that much of the phosphorylation occurs in the 10 serines immediately C-terminal to the helix-loop-helix, and it requires IKKγ.

To confirm that the band shift seen in IKKβ when co-expressed with IKKγ was caused by phosphorylation, extracts were treated with alkaline phosphatase. IKKβ co-expressed with IKKγ migrates slower compared with IKKβ (expressed alone) on SDS-PAGE (Fig. 2B, compare lanes 1 and 3). However, after treatment with alkaline phosphatase the IKKβ expressed with IKKγ migrates at the same rate as IKKβ expressed alone (Fig. 2B, lane 4), suggesting that the band shift was caused by phosphorylation. The migration of IKKβM10 (with or without IKKγ) (Fig. 2B, lanes 5–8) was unaffected by the alkaline phosphatase treatment. This indicates that the extent of phosphorylation of IKKβM10 mutant is significantly lower than wild-type IKKβ. It further indicates that much of the phosphorylation observed during co-expression of IKKγ with IKKβ must occur in the 10 serines immediately following the HLH domain.

The alkaline phosphatase experiment also indicates that IKKγ is phosphorylated. IKKγ is seen as a doublet when co-expressed with either IKKβ or IKKβM10; the size of the bands is similar for either complex. Treatment of IKKβ+/γ or IKKβM10+/γ with alkaline phosphatase caused IKKγ to migrate predominantly as the faster migrating band, indicating that the slower migrating band is phosphorylated.

To further demonstrate that the C terminus of IKKβ is phosphorylated and the majority of the phosphorylation occurs on the ten serines
between the HLH and the γBD, we labeled wild type, IKKβ-γBDAA, and IKKβM12 in yeast with [32P]orthophosphate. Wild-type IKKβ and IKKβ-γBDAA were expressed with or without IKKγ and IKKβM12 was expressed with IKKγ (Fig. 2C). The IKKβs were immunoprecipitated using HA antibodies, gel-purified, and cleaved by CNBr for phosphopeptide mapping as described (1). As shown in Fig. 2C, top panel, similar levels of IKKβs were immunoprecipitated. Wild-type and IKKβ-γBDAA are phosphorylated weakly when expressed alone (Fig. 2C, middle panel). Their level of phosphorylation increases significantly when expressed with IKKγ. The phosphorylation of the IKKβM12 mutant in the presence of IKKγ is low and similar to the levels of wild type IKKβ-γBDAA in the absence of IKKγ expression. When co-expressed with IKKγ, the C-terminal fragment of IKKβ, the [32P]-labeled peptide band above the 5-kDa marker is seen in the wild type and IKKβ-γBDAA, but not in IKKβM12 mutant (Fig. 2C, bottom panel).

We further assessed whether IKKβ purified from S9 cells is capable of phosphorylating a peptide containing the C-terminal serines of IKKβ in trans. The entire C-terminal region of IKKβ including the HLH, serine-rich, and the NBD/γBDs were produced with a Myc tag and a Hisα tag at their C terminus in bacteria. For some of these peptides, ten or more of the serines were mutated to alamines. For M10, the first 10 serines after the HLH were substituted with alanine. For M12, twelve serines, including those in and near the NBD/γBD, were substituted with alanine. For M14, 14 serines were substituted with alanine. These peptides were expressed in bacteria and purified using nickel beads.

As shown in Fig. 2D, purified IKKβ was able to phosphorylate the peptide containing the wild type and the M10, M12, and M14 peptides. The mutant peptides were phosphorylated 3–5 times less efficiently than the wild-type peptide. As a control, purified IKKβ strongly phosphorylated its known substrate IκBa. The phosphorylation of the C-terminal peptides by IKKβ was significantly weaker than phosphorylation of the IκBa. The data indicate that IKKβ can phosphorylate its C terminus. However, perhaps caused by the absence of IKKγ and conformational requirements only present within the IKK complex, phosphorylation of the C-terminal serines may not be as specific and efficient in trans. Phosphorylation of M10, M12, and M14 in trans by IKKβ could also occur on threonine residues in the C terminus of IKKβ. Taken together, data in Fig. 2 indicate that the C-terminal serines between HLH and NBD/γBD in IKKβ are autophosphorylated in an IKKγ-dependent manner.

IKKγ Induces Phosphorylation of the T-loop of IKKβ and Mimics a Phosphorylation State of the NBD/γBD That Reduces IKK Activation Significantly—As shown above, autophosphorylation of ten serines between the HLH and NBD/γBD of IKKβ does not seem to play a role in regulating IKK complex activity. May et al. (33) suggested that phosphorylation of serines 740 in the NBD/γBD may down-regulate IKK activity. We used the yeast reconstitution of IKK to examine whether mimicking the phosphorylation state of the serines 740 and 750 in the NBD/γBD has a negative effect in IKKγ-induced activation of IKK complex. Substituting serine to glutamate to mimic a negative charge has resulted in constitutively active MAP kinases (36). In the case of IKK kinases, phosphorylation of the regulatory serines in the T-loop activation region in the kinase domain has resulted in constitutive activity (3). We generated yeast expression vectors for HA-IKKβ-γBD (in which serines 740 and 750 are mutated to alanine, see Fig. 3A). Each IKKβ was expressed alone or with IKKγ on the same vector in yeast, and IKK complexes were partially purified by gel filtration. IKKβ reconstituted with IKKγ (Fig. 3B, lane 2) has higher activity than IKKβ alone (Fig. 3B, lane 1), indicating that IKKγ facilitates self-activation of IKK. Mutating the T-loop regulatory serines to glutamate to mimic the phosphorylated state results in high activity (Fig. 3B, lane 3), which is not further activated by co-expression with IKKγ (Fig. 3B, lane 4). This indicates that the regulatory role of IKKγ is upstream of the phosphorylation of the T-loop serines. Mutation of the T-loop serines to alanines makes the kinase completely inactive (Fig. 3B, lane 5), and this mutated IKKβ T-loop AA cannot be activated at all by the presence of IKKγ (lane 6). When the serines 740 and 750 in the NBD/γBD are mutated to glutamate, IKKβ still has a low level of kinase activity (Fig. 3B, lane 7), similar to wild-type IKKβ (Fig. 3B, lane 1). However, reconstitution with IKKγ does not allow IKKβ-γBD to be activated (lane 8).

4 Y-K. Lee and E. Zandi, unpublished data.
indicates that the analog of phosphorylated serines in the γBD prevents IKKγ from facilitating self-activation of IKK and supports the hypothesis that phosphorylation of these amino acids is a mechanism to maintain IKK in a state of low level of activity. IKKβ with the 6 amino acids in the NBD/γBD domain at the C terminus (LDWSWL) deleted had a low level of activity (Fig. 3B, lane 9), similar to wild-type IKKβ, but IKKγ could not allow the complex to self-activate (Fig. 3B, lane 10). This further indicates that the γBD is required for self-activation of the complex. IKKβ-γBDAA, in which serines at the NBD/γBD (740, 750) were mutated to alanine, had a similar level of IKK activity to wild type (Fig. 3B, lane 11), and IKKγ facilitates its self-activation (Fig. 3B, lane 12) similar to wild type. Finally, when both the T-loop serines and NBD/
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γBD serines are mutated to glutamate to mimic phosphorylation, IKKβ has a high activity (Fig. 3B, lane 13), and its activity is not further enhanced by the presence of IKKγ (Fig. 3B, lane 14). These results indicate that interaction of IKKγ with the NBD/γBD of IKKβ is upstream to the phosphorylation of the T-loop serines, which can occur intramolecularly within the IKK complex.

Gel filtration analyses of the above complexes showed that IKKβ containing point mutations in the NBD/γBD form similar size complexes to the wild type and native IKK from HeLa cells (data not shown). This indicates that mutating serines to alanine or glutamate in the NBD/γBD did not affect IKK complex formation.

To further confirm that mimicking the phosphorylation state of the γBD results in decreased IKK activity in vivo, we examined autophosphorylation of IKKβ in yeast labeled with [32P]orthophosphate. We compared the autophosphorylation of the wild-type, T-loop EE, and γBD EE IKKβ. Yeast were incubated in [32P]orthophosphate for 5 h, induced to synthesize IKKs, harvested, and lysed, immunoprecipitated, separated by SDS-PAGE, and analyzed by autoradiography and Western blot. A representative experiment is shown in Fig. 3C and autophosphorylation data from three independent experiments were normalized based on the signal intensities of corresponding Western blots, and the results are shown in Fig. 3D. The wild-type IKKβ expressed alone was only weakly autophosphorylated (Fig. 3, C and D), but when co-expressed with IKKγ, the level of autophosphorylation increased by 2.3-fold. The IKKβ T-loop EE (which has constitutively high activity toward IkBα, see Fig. 3B) when expressed alone showed similar level of auto-phosphorylated to the wild type. The autophosphorylation of the T-loop EE increased by 3-fold when co-expressed with IKKγ. This result support the data in Fig. 2C that show IKKγ facilitates increased phosphorylation of the C-terminal serines, even under conditions where its presence is not needed to activate IKKβ. The IKKβ T-loop EE mutant cannot be phosphorylated in the T-loop, so this phosphorylation is most likely due to C-terminal phosphorylation, providing further support for the findings shown in Fig. 2. The level of autophosphorylation in IKKβ-γBDEE whose kinase activity toward IkBα is low, even in the presence of IKKγ (Fig. 3B) did not increase much when co-expressed with IKKγ (Fig. 3, C and D). In summary, these data suggest that IKKγ facilitates the C-terminal phosphorylation of IKKβ within the IKK complex. This is true even under conditions where IKKβ is constitutively active. Furthermore, the data support the May et al. (33) findings in that phosphorylation of the NBD/γBD in IKKγ plays a regulatory role in IKKγ-dependent regulation of IKK complex.

Effect of the NBD/γBD Mutations in IKKβ on the Activity of Reconstituted IKK in MEFa−−/−β−−—To investigate whether the data shown in Fig. 3B can be recapitulated in mammalian cells, we generated HA-tagged mammalian expression vectors for IKKβ-γBD and IKKβ-γBDEE. To avoid the interference of the endogenous IKKα or IKKβ, we utilized the MEFa−/−β−− (35). Stable pools of MEFa−/−β−− expressing HA-tagged IKKβ-wt, IKKβ-γBD, or IKKβ-γBDAA were generated, and the activity of IKK complex was examined before and after treatment with IL-1β for 10 min (Fig. A4). Using anti-IKKγ antibody, IKKβ-IKKγ complexes were isolated and the kinase activity was assessed by immune kinase complex assay. The activity of IKKβ-wt was induced by IL-1β as expected (Fig. A4A). The IKKβ-γBD has a similar basal level of activity like the wild-type IKK, but its activity is not induced by IL-1β (Fig. A4A). This is consistent with the yeast experiments in Fig. 3B, and supports the hypothesis that a negative charge(s) in the NBD/γBD of IKKβ can prevent induction of IKK. Preventing the phosphorylation of the serines in the NBD/γBD should allow constitutive activity of the complex, at least to some degree. IKKβ-γBDAA has significantly higher basal level kinase activity compared with the wild type, and this activity is only marginally induced by IL-1β (Fig. A4A). It is important to note that in the presence of endogenous IKKα similar mutations in the NBD/γBD of IKKβ did not allow constitutive activity of the complex (see Fig. 1A), though the activation was faster. This indicates that a wild-type NBD/γBD in one of the kinase subunits within the IKK complex could be sufficient to allow IKKγ to regulate the basal level of IKK complex.

To compare the in vivo activities of wild-type and mutant IKKβs in the cell lines, we determined the phosphorylation of endogenous IkBa using phospho-IkBα antibodies before and after treatment with IL-1β for 5 and 10 min (Fig. B4B). Because phosphorylated IkBa is ubiquitinated and degraded rapidly, proteasome was inhibited by treating the cells with MG-132 (5 μM) for 30 min prior treatment with IL-1β (2 ng/ml) for indicated times in minutes. For immunoblot analysis, 30 μg of whole cell extracts were used. Levels of HA-IKKβ was determined using anti-HA (top panel), phosphorylated IkBa was determined using the phospho-IkBα antibody (Cell Signaling) (middle panel). Total IkBa protein levels was determined using an IkBa antibody (Cell Signaling) (bottom panel).
In cells expressing the IKKβ-γBDAA and not treated with IL-1, phosphorylation of IkBa is significantly higher than the wild-type IKK (Fig. 4B). Treatment of these cells with IL-1 did not increase phosphorylation of IkBa further (Fig. 4B).

Taken together, the data in Fig. 4 support the hypothesis that phosphorylation of the NBD/BD in IKKβ and possibly in IKKα plays a role in regulation of the IKK complex.

**DISCUSSION**

There are at least 16 serine residues in IKKβ, which may be involved in up-regulating and down-regulating IKK activity. These consist of two serine residues in the T-loop and 14 serines following the HLH domain, including 2 serines in and adjacent to the NBD/BD. NIK activates IKKα by direct phosphorylation of T-loop serines (19). However, many other stimuli activate IKK via IKKγ and may not act by direct phosphorylation of T-loop serines. Rather, many stimuli may act by altering the interaction of IKKβ with the NBD/BD at the C terminus of IKKβ. As shown in Fig. 3B, when the serine residues in the NBD/BD of IKKβ are mutated to resemble phosphoserine, the kinase is no longer stimulated by IKKγ in the yeast reconstitution system. Expression of a similar IKKβγBDEE in MEFα−/−β−/− could only be weakly stimulated by IL-1. Furthermore, IKKβγBDAA (where NBD/BD serines cannot be phosphorylated) was either constitutively active or showed more rapid activation kinetics than wild-type IKK. Altogether, these data suggest that phosphorylation of NBD/BD serines may be a mechanism by which IKK is maintained in a low activity state, and upstream stimuli may regulate IKK by altering the phosphorylation status of NBD/BD serines. Phosphorylation of serines in the region where IKKγ binds to IKKβ may alter the interaction between the two subunits, thereby altering its capacity to become activated.

Data presented here and previously (33) indicate that phosphorylation of the NBD/BD of IKKβ maintains IKK in a basal state, but it is unclear what kinase is responsible for catalyzing this phosphorylation. The results of this study demonstrate that IKKβ can phosphorylate a peptide substrate containing the serines in the NBD/BD of IKKβ, indicating that indeed IKKβ can phosphorylate its NBD/BD. However, this phosphorylation is relatively weak (compared with the activity of IKK toward IκB, see Fig. 2D). Perhaps the level of phosphorylation observed is low because IKKβ is phosphorylating exogenous C termini, and the kinase normally autophosphorylates its NBD/BD within the same complex. Perhaps this phosphorylation is weak because IKKγ is needed for this autophosphorylation. Alternatively, it is possible that while IKKβ can indeed autophosphorylate its NBD/BD, in mammalian cells there exists another kinase whose role is to phosphorylate the NBD/BD of IKKβ.

It is clear that phosphorylating the NBD/BD serines can diminish IKK activity, but these are not the only serines phosphorylated in the C terminus of IKKβ. As in mammalian cells, the 10 serines immediately following the HLH are heavily phosphorylated in the yeast reconstitution system. This study indicates that this is due to autophosphorylation, requires active IKKβ, and is strongly enhanced by IKKγ. Previous work using mammalian cells indicated that inactivating mutations in
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the kinase domain (K44A) or T-loop (S177A and S181A) of IKKβ completely prevented IKKβ phosphorylation at the C terminus, suggesting that the mechanism of C-terminal phosphorylation is autophosphorylation (1). However, it remained plausible that only native, active IKK could be recognizable by a putative exogenous IKK C-terminal kinase. Co-expression of IKKγ with IKKβ in the yeast system resulted in increased phosphorylation of IKKβ, primarily in the 10 serines immediately following the HLH as evidenced by direct in vivo labeling as well as by band shift during gel electrophoresis (Figs. 2 and 3). This result is not surprising since expression of IKKγ is known to activate IKKβ, and IKKβ has been shown to autophosphorylate its C terminus. However, when IKKβ is made constitutively active by mutation of T-loop serines to glutamic acids, it is not phosphorylated very strongly. In contrast, when this same form of IKKβ T-loop EE is co-expressed with IKKγ, a strong phosphorylation of the C terminus is seen. This result indicates that while having active kinase is sufficient for IKKβ autophosphorylation, IKKγ is somehow involved in augmenting the phosphorylation of C-terminal serines beyond the HLH. As shown in Fig. 1, phosphorylation of these serines does not appear to affect IKK activity, nor does it appear to have a previously reported down-regulatory function (1).

In studies involving mammalian cells, mutation of T-loop serines to glutamic acids resulted in constitutively high activity (1, 3). However, these cells have an NF-κB activation pathway, it was possible that these phosphorylated residues attracted other molecules to the IKK complex, resulting in activation. Yeast cells lack the NF-κB system (and presumably other factors involved in activating IKK). Nonetheless, when IKKβ with phospho-mimicking residues in the T-loop is expressed in yeast (Fig. 3B), it is constitutively active and does not require IKKγ. Mutation of the T-loop serines to alanines makes the kinase inactive, even in the presence of IKKγ. When both the NBD/yBD serines and the T-loop serines were mutated to glutamic acids (Fig. 3B), the kinase was constitutively active (with or without co-expression with IKKγ). This indicates that phosphorylation of the T-loop serines is the dominant factor governing whether IKKβ is active or inactive; if the T-loop serines are phosphorylated, the kinase is not affected by changes in the phosphorylation status of the C terminus.

These data suggest that there are possibly dual mechanisms for activating IKK (see Fig. 5). Certain pathways (such as NIK) may activate IKK by directly phosphorylating the T-loop serines. Other stimuli may act via IKKγ and the NBD/yBD to cause conformational changes in the IKK complex, facilitating autophosphorylation of T-loop serines and increasing kinase activity. Phosphorylation of the NBD/yBD serines may prevent IKKγ from facilitating autophosphorylation and self-activation during periods of basal activity.

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