Differential Phosphorylation of the Signal-responsive Domain of IκBα and IκBβ by IκB Kinases*

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NF-κB activity is regulated by its association with the inhibitory IκB proteins, among which IκBα and IκBβ are the most abundant. IκB proteins are widely expressed in different cells and tissues and bind to similar combinations of NF-κB proteins. The degradation of IκB proteins allows nuclear translocation of NF-κB and hence plays a critical role in NF-κB activation. Previous studies have demonstrated that, although both IκB proteins are phosphorylated by the same IκB kinase (IKK) complex, and their ubiquitination and degradation following phosphorylation are carried out by the same ubiquitination/degradation machinery, their kinetics of degradation are quite different. To better understand the underlying mechanism of the differences in degradation kinetics, we have carried out a systematic, comparative analysis of the phosphorylation sites of the IκB proteins. We found that, whereas IKKe is a weak kinase for the N-terminal serines of both IκB isoforms, IKKβ is an efficient kinase for those residues in IκBα. However, IKKβ phosphorylates the N-terminal serines of IκBβ far less efficiently, thereby providing an explanation for the slower rate of degradation observed for IκBβ. Mutational analysis indicated that the regions around the two N-terminal serines collectively influence the relative phosphorylation efficiency, and no individual residue is critical. These findings provide the first systematic analysis of the ability of IκBα and IκBβ to serve as substrates for IKKs and help provide a possible explanation for the differential degradation kinetics of IκBα and IκBβ.

In most resting cells, the transcription factor NF-κB is kept dormant in the cytoplasm through its interaction with IκB proteins. There are several IκB isoforms, among which IκBα and IκBβ are the most abundant and well studied (for reviews, see Refs. 1 and 2). IκBα and IκBβ share a common overall domain structure: an N-terminal signal-responsive domain, a central ankyrin repeat domain, and a C-terminal PEST domain. The two IκB proteins also have similar three-dimensional structures as revealed in crystallographic studies on NF-κB-IκB complexes (3–5). In addition, both IκB proteins bind predominantly to NF-κB p65/p50 heterodimers in vivo (6, 7).

However, these two IκB proteins display different degradation kinetics in response to stimulation with NF-κB inducers (6, 8). For example, upon TNF-α treatment, IκBα undergoes rapid and complete degradation (within <15 min) before reappearing due to NF-κB-induced resynthesis of IκBα mRNA. In contrast, the degradation of IκBβ occurs more slowly, with complete degradation occurring 30–60 min following stimulation (6, 8). However, the kinetics of IκBβ degradation varies in different cell types and depends on the nature of the inducer. Thus, whereas the level of IκBβ protein is unaltered in 70Z/3, Jurkat, or EL-4 cells treated with TNF-α (6, 8), IκBβ is partially degraded when NIH3T3 cells are stimulated over extended periods of time by TNF-α (this study). In certain cell lines such as 70Z/3, inducers such as lipopolysaccharide and interleukin-1 trigger complete IκBβ degradation, although the kinetics of degradation is still significantly slower than that of IκBα (6, 8). Although it has been suggested that the rapid activation of NF-κB is due to the rapid degradation of IκBα, whereas a prolonged activation of NF-κB occurs through the less efficient degradation of IκBβ (6, 9–12), the mechanism responsible for the different rates of degradation of the two IκB proteins has remained elusive.

A critical step in the activation of NF-κB is the phosphorylation of IκB proteins by the IκB kinase (IKK) complex. Characterization of the 700–900-kDa IKK complex has led to the identification of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ/NEMO (13–19). IKKα and IKKβ dimerize via their leucine zipper motifs while binding to IKKγ/NEMO with the NEMO-binding domain to form the core IKK complex (17, 20, 21). The IKK complex may also include other components such as a kinase-specific chaperone (Cdcl57/HSP90) that appears to play a role in shuttling the complex from the cytoplasm to the membrane during TNF-α-induced NF-κB activation (22).

Both in vitro and in vivo studies have proven that IKKα and IKKβ can phosphorylate IκB proteins at specific serine residues, including Ser23 and Ser28 of IκBα and Ser19 and Ser23 of IκBβ (13, 14, 16). Phosphorylation at these conserved serine residues leads to ubiquitination and degradation of the IκB proteins. Although it is generally accepted that IKK phosphorylates both IκBα and IκBβ, the efficiency of the two IκB isoforms as substrates has never been carefully examined. Because IKK activity is likely rate-limiting for IκB degradation and NF-κB activation, since a 2-fold reduction in IKKβ causes nearly 80% decrease in NF-κB activation (23), we compared the phosphorylation of IκBα and IκBβ by IKKα and IKKβ in an attempt to better understand the mechanism of differential regulation of IκB degradation.

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The abbreviations used are: TNF-α, tumor necrosis factor-α; IKK, IκB kinase; GST, glutathione S-transferase.
A. 70Z/3

TNFα

0 15 30 60 120 180 min

IκBα

IκBβ

+CHX

IκBα

IκBβ

B. NIH3T3

LPS

0 15 30 60 120 180 min

IκBα

IκBβ

+CHX

FIG. 1. IκB degradation kinetics in response to TNF/ lipopolysaccharide treatment. A and B, left panels, 70Z/3 and NIH3T3 cells, respectively, were treated with TNF-α (10 ng/ml) for the indicated times. Cellular IκBα and IκBβ protein levels were examined by Western blotting. As indicated, cells were treated with cycloheximide (+CHX; 10 μg/ml) for 1 h before TNF-α was added. Right panels, 70Z/3 and NIH3T3 cells, respectively, were treated with lipopolysaccharide (LPS; 10 μg/ml) for the indicated times. Cellular IκBα and IκBβ protein levels were examined by Western blotting.

EXPERIMENTAL PROCEDURES

Plasmids, Cells, and Antibodies—Vector pGEX6P1 (Amersham Biosciences) was used to generate all GST-tagged wild-type and mutant IκB constructs. IKKβ was cloned into pDNA3 (Clontech), and pEF-IκBα and pEF-IκBβ (the pEF vector was from Invitrogen) were used in transfection experiments. HEK293, NIH3T3, IκBα−/−3T3, and IκBβ−/−3T3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. IκBα−/−3T3 and IκBβ−/−3T3 cells were generated by targeted gene deletion. pGEX-IκBα and pEF-IκBβ were purchased from Sigma. All antibodies were purchased from Santa Cruz Biotechnology.

Site-directed Mutagenesis—The pGEX-IκBαN deletion mutant was generated by PCR using specific primers for the 5′- and 3′-terminal sequences. pGEX-IκBαN point mutations were generated by PCR according to point mutagenesis protocol from Clontech. Escherichia coli DH5α bacterial cells were used for cloning, and the mutants were verified by sequencing.

Preparation of GST-tagged Proteins—Plasmids were transformed into BL21 bacterial cells and grown in ampicillin-containing LB medium to an A600 of 0.6. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM to induce production of GST-tagged proteins. The cultures were grown overnight at room temperature. Cells were collected and resuspended in phosphate-buffered saline plus protease inhibitors and then sonicated using a Vibronic sonicator (Virtis). The supernatant was clarified by centrifugation in a microcentrifuge at 14,000 rpm for 30 min and incubated with glutathione-conjugated agarose beads for 30 min. GST-tagged proteins were eluted by 10 mM glutathione in 50 mM Tris buffer (pH 8.0) and dialyzed in 50 mM Tris buffer (pH 8.0).

Transfection—Cells were grown in 10-cm plates to 40% confluence and transfected with the indicated DNAs using FuGENEmix (Roche Applied Science). For transient transfections, cells were harvested after 36–48 h. For stable transfections, puromycin (1 μg/ml final concentration) was added to the medium. Resistant colonies formed after 1 week and were moved to 24-well plates to expand to single clones. Positive stable transfectants were verified for expression of the cloned proteins using specific antibodies.

Kinase Assay—293 cells were treated with TNF-α for 10 min or transfected with the indicated IKK constructs for 48 h before harvesting. Cells were lysed in buffer containing 0.1% Triton X-100, a mixture of protease inhibitors (Roche Applied Science), and phosphatase inhibitors. Supernatants were collected and incubated with anti-IκK polyclonal antibody with protein G-Sepharose beads or anti-FLAG antibody M2-agarose beads for 3 h. The beads were washed with TNT buffer (20 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 1% Triton X-100) and phosphate-buffered saline, before incubation with kinase reaction buffer containing [γ-32P]ATP and the indicated GST-substrate IκB protein for the indicated times. The reaction was stopped by dilution in TNT buffer, followed by pull-down of the GST-tagged proteins using glutathione-conjugated agarose beads. The pelleted beads were washed three times with TNT buffer, and the samples were analyzed by SDS-PAGE. The gels were fixed and vacuum-dried before exposure to BioMax films (Eastman Kodak Co.). After exposure, the gels were rehydrated and stained with Coomassie Blue.

Immunoprecipitation and Immunoblotting—Cells were lysed in TNT buffer supplemented with protease inhibitors. In immunoprecipitation experiments, cell lysates were incubated with 30 μl of anti-IκB antibody M2 affinity gel for 3 h at 4 °C. Immobilized immunocomplexes were washed with TNT buffer three times, boiled in SDS loading buffer, and resolved by 10% SDS-PAGE. Proteins were transferred to Immobilon transfer membrane (Millipore Corp.) and blotted with the indicated primary antibodies for 3 h at room temperature, followed by the appropriate secondary antibody for 1 h. Immunoreactive bands were visualized by ECL.

RESULTS

Differential Degradation Kinetics of IκBα and IκBβ—When cells such as the mouse pre-B cell line 70Z/3 are treated with TNF-α, the cytosolic protein levels of IκBα and IκBβ change with distinct patterns (6, 8, 9). Whereas IκBα disappears and reappears over a period of 3 h, IκBβ levels remain unaltered. Activated NF-κB induces the resynthesis of IκBα via the three κB sites in the IκBα promoter (24–28). Although the IκBβ promoter region harbors a single κB site, it is minimally responsive to NF-κB binding (29). In the presence of cycloheximide, IκBα did not reappear after 30 min, whereas IκBβ levels were unaffected (Fig. 1A, left panel), thus excluding the possibility that IκBβ protein levels were maintained through a balance of degradation/turnover and resynthesis. The non-responsiveness of IκBβ to TNF-α treatment was, however, cell
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**Fig. 2.** The IKK complex differentially phosphorylates IκBα and IκBβ.

A, 293 cells were transfected with TNF-α (10 ng/ml) for 10 min (+) or left untreated (−) before harvesting. Endogenous IKK complex was purified using anti-NEMO polyclonal antibody and then incubated with GST-tagged full-length IκBα and IκBβ in kinase reaction buffer. GST-IκB proteins were then pulled down with glutathione-conjugated agarose, resolved by SDS-PAGE, fixed, and exposed to film (kinase activity (KA)). Equal amounts of GST-IκB proteins were used in the experiment, as shown by Coomassie Blue (CB) staining of the gel. The lower band in all lanes of the Coomassie Blue-stained panel marked with an asterisk represents the GST fragment alone (~28 kDa). B and C, 293 cells were transfected with constitutively active FLAG-fusion (F)-IKKα(CA) or FLAG-IKKβ(CA), respectively, or empty vector (−) and incubated for 36 h before harvesting. The IKK complex was purified using anti-FLAG monoclonal antibody M2 and then incubated with GST-tagged full-length IκBα and IκBβ in the kinase assay. The band at ~97 kDa represents autophosphorylated FLAG-tagged IKKα(CA) or IKKβ(CA). The phosphorylation of the IκBα substrate seen in lane 1 in B is probably due to immunoprecipitation of some endogenous IKK from the untransfected cells by the FLAG-conjugated beads.

![Image](http://www.jbc.org/)

Type specific. In NIH3T3 cells, IκBβ did respond to TNF-α, although the rate and extent of degradation were noticeably less than those of IκBα (Fig. 1B, left panel). When both cell types were treated with lipopolysaccharide, the level of IκBβ degradation was greater, but the overall pattern remained the same (Fig. 1, A and B, right panels).

The IKK Complex Differentially Phosphorylates IκBα and IκBβ—Because phosphorylation is the first step that marks IκB proteins for degradation, we examined whether IκBα and IκBβ can be phosphorylated equally well by the IKK complex. We therefore performed in vitro kinase assays using recombinant GST-IκBα and GST-IκBβ proteins as substrates and immunoprecipitated the IKK complex from TNF-α-stimulated HEK293 cells as the kinase. It has been previously shown that, upon stimulation by TNF-α, IKK activity peaks within 5–15 min before declining to ~25% of its peak value by 30 min (15, 16, 30) (data not shown). We therefore used 293 cells stimulated with TNF-α for 10 min as a source of active IKK in our assays. Since IKKγ/NEMO exists as a complex with both IKKα and IKKβ in vivo (17), we immunoprecipitated the IKK complex using anti-NEMO polyclonal antibody. Surprisingly, we observed a significant difference in the level of phosphorylation of IκBα and IκBβ (Fig. 2A, upper panel), even though the amount of GST-IκB proteins was similar (lower panel). The IKK complex immunoprecipitated from unstimulated cells also phosphorylated the IκB proteins weakly (Fig. 2A, lanes 1 and 3), indicating that some constitutive activation of IKK occurred in these cells.

Although both IKKα and IKKβ can phosphorylate the N-terminal serine residues in IκB proteins, we wanted to test whether they exhibit any preference for IκBα or IκBβ. Both IKKα and IKKβ contain an activation loop, and phosphorylation of specific serine residues in the activation loop leads to activation of the kinases (14, 31, 32). It has been shown that substitution of the single residues (Ser32 and Ser36 in IKKα and Ser177 and Ser181 in IKKβ) with glutamic acid (which mimics phosphoserine) results in constitutively active IKKαs and IKKβ (14). We therefore transfected constitutively active FLAG-tagged IKKαs and IKKβs into 293 cells and immunoprecipitated them using anti-FLAG antibody-conjugated agarose beads. The cloning vector was used as a negative control (labeled as − in the figures). The majority of the overexpressed IKKα or IKKβ formed homodimers, with very small amounts of heterodimers being formed between transfected and endogenous IKK subunits (data not shown). As shown in Fig. 2 (B and C), both constitutively active IKKαs and IKKβ homodimers phosphorylated IκBα more efficiently than IκBβ, as seen previously for the endogenous TNF-α-stimulated IKK complex (Fig. 2A).

**IKKβ Differentially Phosphorylates IκBα and IκBβ N Terminals—**Some prior reports have indicated that, besides Ser32 and Ser36 in IκBα and Ser177 and Ser181 in IκBβ, the IKK complex can also phosphorylate the C terminus of IκBα (16, 33). To check whether the differential phosphorylation between full-length IκBα and IκBβ is due to additional C-terminal sites of phosphorylation in IκBα, we compared the efficiency of phosphorylation of full-length IκBα and IκBβ versus truncated IκB proteins containing only the N-terminal serine residues (IκBαN containing the N-terminal 54 residues and IκBβN containing the N-terminal 44 residues). Using TNF-α-activated IKK immunoprecipitated with anti-NEMO antibody, we found no significant difference in the efficiency of phosphorylation between the full-length and N-terminal IκBα and IκBβ substrates (Fig. 3A).

We then tested whether there is any difference in the ability of IKKα and IKKβ to phosphorylate full-length IκB proteins...
and their N-terminal portions. As shown in Fig. 3B, whereas IKKβ (constitutively active) homodimers strongly phosphorylated both full-length IκBα and IκBβ N, the equivalent IκBβ constructs were phosphorylated poorly. In contrast, whereas IKKα (constitutively active) homodimers were able to efficiently phosphorylate both full-length IκBα constructs, it was unable to phosphorylate IκBα N and IκBβ N. To determine whether the inability of IKKα to phosphorylate the IκB N terminus might be influenced by IKKα/NEMO, the regulatory subunit of the IKK complex, we transfected wild-type IKKα with or without cotransfection of NEMO to generate the kinase used to phosphorylate IκBα and IκBβ N termini. However, in both cases, overexpressed wild-type IKKα efficiently phosphorylated full-length IκBα and, to a lesser extent, full-length IκBβ, but was unable to phosphorylate the N-terminal IκB substrates (Fig. 3C).

Comparison of the Phosphorylation and Degradation Kinetics of IκB Proteins—To explore the possibility that inefficient IκBβ phosphorylation by IKKβ may be responsible for the slower and incomplete degradation of IκBβ, we compared the kinetics of IκBα and IκBβ N phosphorylation by IKKβ. As mentioned above, the activity of IKKβ in most cell lines treated with TNF-α peaks between 5 and 15 min and then decreases significantly, most likely due to extensive autophosphorylation of its C-terminal region (30). As described above, we used immunoprecipitated IKKβ(CA) and incubated it with GST-IκBα N (amino acids 1–54) and GST-IκBβ N (amino acids 1–44) in kinase reaction buffer. The phosphorylation of endogenous NEMO in immunoprecipitations of NEMO from TNF-α-stimulated cells (lanes 2 and 4) was variable. KA, kinase activity; CB, Coomassie Blue. GST-tagged full-length IκBα and IκBβ, GST-IκBα N, and GST-IκBβ N were used as substrates for overexpressed IKKβ(CA) (left panels) and IKKα(CA) (right panels) in the kinase assay. The autophosphorylation of IKK and the phosphorylation of NEMO by IKK, enhanced by TNF-α treatment, are labeled on the autoradiograph accordingly. C, FLAG (F)-tagged wild-type IKKα was transfected in 293 cells and used as kinase source for GST-tagged full-length IκBα and IκBβ, GST-IκBα N, and GST-IκBβ N in the kinase assay (left panel). Wild-type IKKα was cotransfected with or without NEMO in 293 cells and used to phosphorylate GST-IκBα N and GST-IκBβ N in the kinase assay (right panel).
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over time (Fig. 4A). Whereas the phosphorylation of both IκBα and IκBβ also increased over time, the dramatic difference between IκBα and IκBβ remained even after 1 h of incubation with IKKβ (Fig. 4A). Quantitation of the degree of phosphorylation by a PhosphorImager indicated a roughly linear correlation between time and degree of phosphorylation for both IκBαN and IκBβN. We also noticed that the phosphorylation of IκBβ at 1 h was less than that of IκBα at 5 min. Interestingly, the level of IκBβ phosphorylation increased only slightly even after 3 h of incubation (data not shown). These results suggest that the inefficiency of IκBβ phosphorylation by IKKβ cannot be explained as a simple kinetic difference and probably reflects a more fundamental difference in the ability of the two proteins to serve as substrates for IKKβ.

Differential Phosphorylation of IκB N Termini Is Linked to Protein Stability of IκB Proteins—To further establish the link between phosphorylation efficiency of IκB proteins and their degradation pattern, we transiently transfected HeLa cells with IκBα and IKKβ. Cotransfection of IKKβ(CA) dramatically decreased the protein level of wild-type IκBα, but did not affect the level of the non-phosphorylatable IκBαAA mutant, in which the two conserved serine residues at positions 32 and 36 had been mutated to alanines. Therefore, this experiment establishes that the instability of IκBα protein caused by IKKβ cotransfection was dependent on phosphorylation of Ser32 and Ser36. However, cotransfection of IKKβ did not affect the protein stability of IκBβ, which likely correlates with the inability of IKKβ to efficiently phosphorylate IκBβ (Fig. 5B, lower panel, third and fourth lanes). To further establish the connection between phosphorylation efficiency and stability of transfected IκB proteins, we generated two chimeric proteins, IκBαβ and IκBβα, by swapping the N-terminal signal peptide region between IκBα and IκBβ (Fig. 5B, upper panel). Because the N termini of IκB proteins determine the efficiency of phosphorylation by IKKβ, GST-IκBαβ was efficiently phosphorylated in in vitro kinase assays, whereas GST-IκBβα was not (data not shown). Consistent with this difference in phosphorylation efficiency, the level of transfected IκBαβ protein was significantly reduced by IKKβ cotransfection, whereas the level of IκBβα was affected only marginally. Thus, the N termini of IκB proteins determine their ability to be phosphorylated by IKKβ, which in turn is reflected in their rate of degradation.

Residues 28–42 in IκBα and Residues 15–31 in IκBβ Influence Substrate Phosphorylation Efficiency—To determine which specific features of the IκBα N terminus sequence make it a better substrate for IKKβ, we carried out a comprehensive mutational analysis of the N-terminal regions of IκBα and IκBβ. We initially focused on the first 12 amino acids in IκBα, which is a sequence unique to IκBα (Fig. 6A). We generated an IκBαN–12 mutant by deleting these 12 amino acids and compared the phosphorylation efficiency of this mutant and the wild-type IκBα proteins. In vitro kinase assays revealed no significant difference between this mutant and wild-type IκBα, indicating that these 12 unique residues of IκBα do not determine the phosphorylation efficiency of IκBα proteins (Fig. 6B).

We then divided the remaining homologous region in the N termini of IκBα proteins (residues 13–54 in IκBα and residues 1–42 in IκBβ) into three parts and swapped the different parts between IκBα and IκBβ to produce a set of chimeric GST-tagged N-terminal IκBα proteins, IκBαNαβα, IκBαNβαβ, IκBαNβαα, IκBαNαβα, IκBαNαββ, and IκBαNαβ (Fig. 6C). Only one of the GST-IκBαN chimeric proteins, GST-IκBαNαβα, was phosphorylated to the same level as GST-IκBαN in in vitro kinase assays (Fig. 6D). Because both IκBαNαββ and IκBαNββ were poorly phosphorylated by IKKβ, we believe that sequence elements present in residues 28–54 of IκBα are responsible for the high phosphorylation efficiency of IκBα.

To further narrow down the sequences responsible for determining the efficiency of IκBα as a substrate for IKKβ, we made additional chimeras by fusing different lengths of the sequence from residues 39 to 54 of IκBα with the central core of amino acids 29–38 from IκBα (data not shown). Analysis of these chimeras indicated that attaching residues 29–43 to the central core in the IκBαNαββ chimera (where residues 29–43 are derived from IκBα, with the flanking sequences from IκBβ) allowed it to be phosphorylated as efficiently as IκBαN (Fig. 6, E and F, third lane). Since residues 29–43 of IκBα appear to be the critical sequence responsible for efficient phosphorylation of IκBα, we generated a set of IκBαN mutants by replacing smaller clusters of residues in this region with their counter-
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In a continuing effort to understand the basis for the differential kinetics of IκBα and IκBβ degradation, we have been searching for cis- and trans-acting factors that might influence the behavior of the two IκB isoforms (6, 34). The findings reported in this study suggest that the amino acid sequence of the N-terminal region that includes the actual sites of phosphorylation determines the efficiency with which the two IκB proteins can be phosphorylated by IKKβ. This intrinsic difference in phosphorylation efficiency would therefore likely contribute to the differential kinetics of degradation of the two isoforms observed in response to some inducers in some cells (6, 8). It is important to note that this difference is unlikely to fully explain all observations regarding differential regulation of these isoforms. In particular, it has been reported that, whereas IκBβ is degraded in response to TNF signaling in some cells, it is unaffected in many other cells (6, 8). Such variability in the behavior of IκBβ in different cells is difficult to explain on the basis of intrinsic differences in the ability of IKKβ to phosphorylate IκBα and IκBβ. It is also not completely clear how the differences between IκBα and IκBβ seen in this analysis can be reconciled with the results of IκBβ knock-in mice (35). In that study, a tagged IκBβ was knocked into the IκBα locus, and the finding that IκBβ in these cells rescued the lethality seen in IκBα knockout mice suggested that IκBα and IκBβ are interchangeable and that their differential responses to NF-κB inducers are not due to intrinsic differences in the proteins (35). It is likely that trans-acting factors such as κB-Ras proteins also contribute to the observed differences in the degradation kinetics of the two IκB isoforms since κB-Ras binds preferentially to IκBβ in cells (34, 36).2

We observed a surprising and unexpected difference in the ability of IKKα to phosphorylate full-length IκB proteins versus the N-terminal fragments of IκB proteins. Whereas IKKβ was able to phosphorylate both the full-length and truncated IκB substrates equally well, IKKα displayed a strong preference for the full-length proteins and was almost inactive on the N-terminal fragments. The inability of IKKα to efficiently phosphorylate the N-terminal IκB substrates also raises the possibilities that, in vivo, IKKα might not phosphorylate IκB proteins and that the lower level of phosphorylation observed with the full-length IκB proteins as substrates may be an in vitro phenomenon since it is known that many kinases lose their selectivity when tested in vitro at high concentrations. Such an explanation would also predict that IKKβ, rather than IKKα, is responsible for phosphorylating the two conserved serines in IκB proteins and transducing inflammatory signals, such as from TNF-α. This observation corroborates previous genetic studies that have disputed the apparent commonality of the two subunits of IKK (37). IKKα−/− mice died perinatally due to limb and skin abnormalities; however, no impairment in

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2 C. Wu and S. Ghosh, unpublished data.
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IκB degradation in response to signaling by cytokines such as TNF-α and interleukin-1 was observed (38, 39). More recently, further analysis of mice in which a catalytically inactive form of IKKα was knocked in led to the discovery that IKKα functions in different biological processes, including breast development, keratinocyte differentiation, and lymph node development, not all of which are NF-κB-dependent (40–42). In instances where IKKα has been shown to influence NF-κB, the effect appears to be due to an alternate pathway that leads to the activation of p52-ReIB complexes following the processing of the p100 subunit (41). Therefore, it is possible that the inability of IKKα to phosphorylate the N-terminal IκB substrates hints at the pleiotropic nature of IKKα, whose more important targets for phosphorylation might be NF-κB p100, histone H3, and NF-κB p65.

The major finding of this study is, however, the clear demonstration of the difference in the ability of the two IκB isoforms to serve as phosphorylation substrates for IKKβ. Comparison of the sequence around the target serine residues in the N-terminal signal-responsive domains of IκBα and IκBβ indicates the absence of an aspartic acid immediately upstream of the second serine residue in IκBβ (Asp35 in IκBα versus Gly22 in IκBβ). However, as shown in Fig. 6, changing the glycine in IκBβ to aspartic acid did not enhance the ability of IKKβ to phosphorylate the IκBβ protein. Instead, it appears that a larger segment from amino acids 29 to 43 is responsible for allowing efficient phosphorylation of IκBα. Unfortunately, the existing crystal structures of IκB proteins do not include this segment; and therefore, it is difficult to propose a model to explain the role played by this region of IκBα. Ultimately, a co-crystal of the IκB N-terminal domain with IKKβ will be necessary to understand the selectivity displayed by IKKβ for IκBα.

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