A Novel Ubiquitin-like Domain in IkB Kinase β Is Required for Functional Activity of the Kinase*

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Activation of NF-κB requires two highly related kinases named IKKα and IKKβ that share identity in the nature and positioning of their structural domains. Despite their similarity, the kinases are functionally divergent, and we therefore sought to identify any structural features specific for IKKα or IKKβ. We performed bioinformatics analysis, and we identified a region resembling a ubiquitin-like domain (ULD) that exists only in IKKβ and that we named the UBL-like domain (ULD). Deletion of the ULD rendered IKKβ catalytically inactive and unable to induce NF-κB activity, and overexpression of only the ULD dose-dependently inhibited tumor necrosis factor-α-induced NF-κB activity. The ULD could not be functionally replaced within IKKβ by ubiquitin or the corresponding region of IKKα, whereas deletion of the equivalent section of IKKα did not affect its catalytic activity against IκBα or its activation by NF-κB-inducing kinase. We identified five residues conserved among the larger family of UBL-containing proteins and IKKβ, and alanine scanning revealed that the leucine at position 353 (Leu353) is absolutely critical for IKKβ-induced NF-κB activation. Most intriguingly, the L353A mutant was catalytically active but, unlike wild-type IKKβ, formed a stable complex with the NF-κB p65 subunit. Our findings therefore establish the ULD as a critical functional domain specific for IKKβ that might play a role in dissociating IKKβ from p65.

NF-κB1 describes a family of structurally and functionally related, ubiquitously expressed transcription factors that play pivotal roles in innate and adaptive immunity, inflammation, development, cell growth, and survival (1, 2). A defining feature of most NF-κB responses is their inducibility. Thus, NF-κB proteins are normally sequestered inactive in the cytoplasm of resting cells through interaction with distinct members of a family of inhibitory proteins named the IκBs. Following appropriate stimulation, the IκB proteins are degraded, and NF-κB migrates to the nucleus where it binds to specific DNA motifs within the promoters of its target genes (1, 2).

The most intensely studied NF-κB activation pathway is that induced by the pro-inflammatory cytokine tumor necrosis factor (TNF)-α. In response to TNF stimulation, IκB proteins (typified by IκBα) become rapidly phosphorylated on two specific N-terminal serine residues, and this signals their subsequent ubiquitination and proteasomal degradation. Degradation of IκBα liberates NF-κB dimers that most commonly consist of the p65 (RelA) and p50 NF-κB subunits, and these p65:p50 heterodimers regulate the expression of a wide range of genes that include those of pro-inflammatory cytokines, leukocyte adhesion molecules, and anti-apoptotic proteins (2, 3). Arguably, the most important intermediate signaling event in this pathway is the phosphorylation of IκBα, and tremendous effort from a number of laboratories has identified and characterized the signal-responsive kinase complex responsible for this crucial step (4). This catalytic activity resides in a complex of proteins named the IκB kinase (IKK) complex that consists of three core subunits named IKKα (IKK1), IKKβ (IKK2), and NF-κB essential modulator (NEMO) that is also known as IKKγ (5–9). A number of elegant genetic studies have clearly demonstrated that phosphorylation of IκBα in response to TNFα signaling is absolutely dependent upon IKKβ and NEMO (10–16), and this pathway that results in liberation of mainly p50:p65 heterodimers is now named the “classical” NF-κB pathway (17). Activation of the classical pathway underlies the vast majority of the known functions of NF-κB in immune and inflammatory responses, cell survival, and development (17).

In contrast to the fundamental role for IKKβ in the classical pathway, IKKα appears for the most part to be functionally redundant (12, 13, 16). Thus IκBα degradation and activation of NF-κB in response to TNFα remains intact in cells derived from IKKα−/− animals, whereas this is completely ablated in both IKKβ−/− and NEMO−/− cells (10–16). However, evidence exists that IKKα plays a role in mediating the classical pathway in response to a subset of inducers including receptor activator of NF-κB ligand (RANKL) (18), and it is known to be able to phosphorylate IκBα in vitro, albeit with a lower relative activity than IKKβ (19). Recently, IKKα has been shown to migrate to the nucleus and to perform an epigenetic function by regulating histone phosphorylation in the vicinity of classical NF-κB-dependent genes (20, 21). In addition, IKKα may play a regulatory role within the IKK complex by trans-phosphorylating IKKβ (22), and loss of IKKα appears to affect the expression of a number of genes activated in response to pro-inflammatory cytokines (23), although the mechanisms responsible for these effects remain unclear. Nevertheless, despite these separate lines of evidence, it remains that the classical NF-κB pathway is absolutely dependent upon IKKβ and that IKKα is dispensable.
sable for IKKβ-dependent IκBα phosphorylation and subsequent NF-κB activation.

In contrast to its lack of function in classical NF-κB signaling, IKKα is the key regulator of a recently described "noncanonical" NF-κB pathway (24–26). In this pathway IKKα specifically phosphorylates the C terminus of the NF-κB p100 subunit (also known as NF-κB2) inducing its ubiquitination and processing, and in a manner analogous with IκBα degradation, p100 processing releases its N-terminal portion (p52) as a transcriptionally active heterodimer with RelB (24–26). This series of events is completely independent of IKKβ and NEMO and functions in IKKβ−/− and NEMO−/− cells (24–26). A critical component of the noncanonical pathway is the upstream kinase NIK (NF-κB-inducing kinase) that phosphorylates and activates IKKα (27), and activation of this pathway is absent in alv/alv mice that carry a mutated NIK gene (24, 27). Studies of alv/alv mice together with accumulated genetic evidence clearly demonstrate that the noncanonical NF-κB pathway is critical for the maturation of B-cells and development of lymphoid organs. Consistent with this, p100 processing only occurs in response to signals from a subset of TNF receptor family members involved in lymphoid organogenesis and B-cell maturation as follows: B-cell activating factor receptor, CD40, and the lymphoxygen-β receptor (24, 25, 28). In turn, the inducers of this pathway are the B-cell activating factor, CD40L, heterotrimeric LTα1β2, and LIGHT that also binds the lymphoxygen-β receptor. Only five genes have been definitively identified as targets of the noncanonical pathway: the cytokine B-cell activating factor and the chemokines SLC (CCL21), ELC (CCL19), BLC (CXCL13), and SDF-1 (CXCL13) (24). Consistent with the genetically defined function of the pathway, these are all involved in either B-cell maturation or the development and function of lymphoid organs.

It is clear from these studies that although they physically interact within the IKK complex, IKKα and IKKβ perform highly distinct functions. Although it has been suggested that IKKα functions in the noncanonical pathway via a separate IKKα-alone complex, such a complex has yet to be described and molecularly characterized (24). Despite this potentially separate complex however, it remains that the IKKs share remarkable structural similarity, and consequently the precise mechanisms that underlie their divergent functions remain unknown. In this regard both kinases possess N-terminal catalytic domains, centrally located leucine zippers through which they interact, and a helix-loop-helix domain that is critical for their activation (29). In addition, IKKα and IKKβ each contain a C-terminal NEMO-binding domain (NBD), which we have demonstrated to facilitate NEMO interaction with both kinases (30, 31). In light of these similarities, we therefore sought to identify any novel domains or sequences within either of the IKKs that might account for their functional divergence.

We describe here the identification and functional characterization of a novel ubiquitin-like domain (ULD) we have identified in IKKβ. Such a domain does not exist in IKKα. We demonstrate that deletion of or mutations within the ULD profoundly affect the function of IKKβ, whereas loss of the identical segment of IKKα does not affect its activity. Furthermore, our findings strongly suggest that the IKKβ ULD plays a fundamental role in regulating the interaction of the IKK complex with p65. We therefore conclude that the ULD is absolutely critical for the induced activity of IKKβ, and we further hypothesize that this novel IKKβ-specific domain contributes to the functional divergence of the IKKs.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—HeLa, HEK293, and COS cells were obtained from ATCC (Manassas, VA) and maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM l-glutamine, penicillin (50 units/ml), and streptomycin (50 μg/ml). Mouse anti-FLAG (M2) and anti-FLAG-coupled agarose beads were purchased from Sigma. Mouse anti-Xpress was purchased from Invitrogen; rabbit anti-p65 (SA-171) was from Bioron (Plymouth Meeting, PA), and the horseradish peroxidase-conjugated secondary antibodies against either rabbit or mouse IgG were both from Amersham Biosciences. Anti-IκBα and anti-glyceraldehyde-3-phosphate dehydrogenase were from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-p100 (NF-κB2) was from Upstate Biotechnology, Inc. Recombinant human TNFα was purchased from R & D Systems (Minneapolis, MN).

**Plasmids and PCR Mutagenesis**—Full-length cDNA clones of human IKKα and IKKβ were the generous gifts from Dr. Michael Karin (University of California, San Diego). All subcloning and mutagenesis procedures were performed by PCR using cloned pFlu DNA polymerase (Invitrogen, La Jolla, CA). All PCR products and primer sequences are available upon request. Wild-type and mutated IKKα cDNAs were inserted between the KpnI and NotI restriction sites of pcDNA-3.1-Xpress (Invitrogen), and all IKKβ cDNAs were inserted into the EcoRI and Xhol sites of the same vector. FLAG-tagged versions of wild-type and mutated IKKα and IKKβ were constructed by subcloning into the EcoRI/Xhol and HindIII/NotI sites within pFLAG-CMV2 (Stratagene, La Jolla, CA). Human IKKα and IKKβ were amplified previously as were the GST-p65N and GST-p65C constructs (32). Full-length cDNAs encoding human ubiquitin and NIK were obtained from HeLa cDNA by PCR cloning using pFlu DNA polymerase. Point mutations within the ULD were made using the QuickChange® site-directed mutagenesis kit from Stratagene. To make all of the domain substitution mutations in IKKα and IKKβ, an EcoRV site was inserted into the IκBα-Ub or IKKα-Ub construct across the join. The fragment mutated was inserted into the kinases were generated by using primers flanked by EcoRV sites, and these were then ligated into the EcoRV-cut ULD constructs.

The GST-ULD fusion protein was constructed by inserting a PCR-generated fragment encompassing the IKKβ ULD (encoding amino acids Leu66 to Met86) between the EcoRI and NotI sites within pGEX-4T1 (Amersham Biosciences). A cDNA encoding human NEMO was obtained as described previously (31). GST-NEMO was constructed by subcloning the full-length cDNA into the EcoRI and XhoI sites of pGEX-4T1. The fusion of GST with the first 90 amino acids of human IκBα (GST-IκBα(1–90)) that was used as a substrate in the kinase assays was described previously (31). GST proteins were made in Escherichia coli (BL21) by treating transformed bacteria with 0.4 mM isopropyl-β-D-thiogalactopyranoside (Sigma) and following the manufacturer's protocol for protein recovery provided with the vector.

**Interaction Analysis**—For GST pull-down analysis, IKKα or IKKβ in pcDNA3.1 were transcribed in vitro and translated in the presence of [35S]methionine using the TNT-T7 Quick system from Promega (Madison, WI). Labeled proteins (1 μl of reticulocyte lysate) were incubated with GST, GST-NEMO, or GST-ULD (1 μl of TNT (50 mM Tris, pH 7.5, 200 mM NaCl, 1% Triton X-100) containing protease inhibitors (Complete Protease Inhibitor Mixture, Roche Diagnostics) at 4 °C for 30 min, and 20 μl of a 50% (w/v) slurry of glutathione-agarose beads (Amersham Biosciences) was then added and incubated a further 15 min. Proteins were then precipitated and washed extensively in TNT before addition of sample buffer (20 μl). Samples were then separated by SDS-PAGE (10%), and the resulting gels were stained with Coomassie Blue, fixed, and then examined autoradiographically.

For transient transfection studies, 1 × 105 COS cells grown in 6-well trays were transfected with 1 μg of total DNA using the FuGENE 6 transfection reagent (Roche Diagnostics). All DNA/FuGENE 6 incubations were performed at a ratio of 1 μg of DNA per 3 μl of FuGENE 6 according to the manufacturer’s recommended protocol in Opti-MEM medium (Invitrogen). After 48 h, cells were lysed in 500 μl of TNT, and then complexes were immunoprecipitated (IP) by using anti-FLAG-coupled agarose beads. A portion of each lysate taken prior to immuno precipitation (5%) was retained for analysis (pre-IP). Precipitated proteins were analyzed by immunoblotting using epitope-specific (anti-FLAG or anti-Xpress) antibodies that were visualized using enhanced chemiluminescence (ECL) reagents from Amersham Biosciences.

**Luciferase Reporter Assay**—Dual luciferase reporter assays were performed essentially as described previously (30, 31). Briefly, 2.5 × 105 HeLa cells grown on 12-well plates were transiently transfected using FuGENE 6 with the NF-κB-dependent reporter construct pBIIx-luc (0.2 μg/well) together with the Renilla luciferase vector (0.02 μg/well). Total DNA concentration in each experiment (1.0 μg/well) was maintained by...
adding the appropriate empty vector to the DNA mixture. Forty-eight hours after transfection, cells were lysed in passive lysis buffer (Promega), and luciferase activity was measured using the dual luciferase assay kit from Promega. In some experiments the levels of transfected proteins in 20 μg of lysates were examined by immunoblotting by using appropriate epitope tag-specific antibodies.

**Immune Complex Kinase Assay**—For immune complex kinase assays, 1 × 10⁶ HeLa cells grown on 6-well plates were transiently transfected with 1 μg of the FLAG-tagged version of the kinase constructs using the FuGENE 6 reagent as described above. Forty-eight hours after transfection, the cells were either untreated or treated with TNFα (10 ng/ml) and then lysed on ice in 500 μl of TNT for 15 min. Protein content in each lysate was determined by using a Bio-Rad protein assay kit (Bio-Rad) and then normalized among the samples. Proteins in lysates were immunoprecipitated using anti-FLAG (M2) coupled agarose beads for 1 h at 4 °C, and the precipitates were washed extensively in TNT and then kinase buffer (20 mM HEPES, pH 7.5, 20 mM MgCl₂, 1 mM EDTA, 2 mM NaF, 2 mM β-glycerophosphate, 1 mM dithiothreitol, 10 μM ATP). Precipitates were then incubated for 15 min at 37 °C in 20 μl of kinase buffer containing appropriate GST-fused substrate proteins and 10 μCi of [γ-³²P]ATP (Amersham Biosciences). The substrate was then precipitated using glutathione-agarose (Amer- sham Biosciences) and washed extensively with TNT. Beads were then suspended in 20 μl of sample buffer, and samples were separated by SDS-PAGE (10%). Kinase activity was determined by autoradiography.

**Sequence Alignment**—All sequence alignments were performed using MacVector software from Accelrys (San Diego, CA).

## RESULTS

**IKKβ Contains a Novel Ubiquitin-like Domain**—We performed a series of proteomic data base searches using various web-based profiles, motifs, and protein family analysis programs to identify any novel structural or functional domains in either IKKa or IKKβ. As expected, the previously described structural features of both kinases, including their N-terminal catalytic domains, central leucine zipper motifs, and C-terminal helix-loop-helix and NEMO-binding domains, were identified through a combination of separate approaches. Most surprisingly, however, analysis of the complete amino acid sequence of human IKKβ using the ExPaSY (Expert Protein Analysis System) molecular biology server and the PROSITE data base (us.expasy.org/cgi-bin/scanprosite) identified a region of 78 amino acids from Leu307 to Met384 corresponding to a region of 78 amino acids from Leu307 to Met384 corresponding to the Ubiquitin_2 (type 2 ubiquitin-like) profile (PROSITE document PD000271; PROSITE accession number PS50053). No such domain was detected in either IKKa or the related kinase IKKe/IKKi.

Despite this sequence identity with ubiquitin, we noted that the PROSITE data base classified this region of IKKβ as a false positive for the Ubiquitin_2 profile, suggesting that it does not belong to the larger family of ubiquitin-like domain-containing proteins per se (us.expasy.org/cgi-bin/nicesite.pl?PS50053). Therefore, we further investigated the IKKβ sequence using the Profilescan Server (hits.isb-sib.ch/cgi-bin/PPSCAN) maintained by the Swiss Institute for Experimental Cancer Research, and this analysis identified the same region as a strong match with a normalized match score of 9.584 (statistical interpretation of match score significance is available at hits.isb-sib.ch/doc/metif_score.shtml). Subsequent search analysis using the Protein Families (Pfam) data base of Alignments and Hidden Markov Models (www.sanger.ac.uk/software/pfam/search.shtml) identified a shorter ubiquitin domain (Pfam document: PF00240) between residues 319 and 354 of IKKβ, and this was also recognized, together with the longer PROSITE Ubiquitin_2 profile, as a ubiquitin domain (document IPR000626) using the Interpro search analysis program of the European Bioinformatics Institute (www.ebi.ac.uk/interpro/index.html). Therefore, we conclude from this accumulated bioinformatic evidence that IKKβ but not IKKe contains a domain located between residues 307 and 384 (more specifically between residues 319 and 354) that displays significant sequence identity with ubiquitin. Although this region strongly resembles both ubiquitin and the UBLs present in a family of unrelated proteins (33), the false positive classification from the original PROSITE search led us to name this region the UBL-like domain (ULD) of IKKβ. The position of the IKKβ ULD and its sequence alignment with human ubiquitin is shown in Fig. 1, A and B. Similar alignment of the corresponding region of IKKa (Ile307–Val384) (Fig. 1C) demonstrates the significantly lower degree of similarity and identity with ubiquitin.

**The IKKβ ULD Is Required for Catalytic Function**—Ubiquitin-like domains have been identified in an expanding family of proteins (33), which include the yeast DNA repair enzyme Rad23 and its human homologues HHR23A and -B, the yeast cell cycle control protein Dsk2, and its human homologues hPLIC-1 and -2, the causative gene of autosomal-recessive juvenile parkinsonism named Parkin, and the anti-apoptotic protein Bag-1 (33). Furthermore, some proteins (i.e. p59 OASL) contain two copies of the domain within their open reading frames (34, 35). The precise role of the UBL within many proteins remains unknown; however, it has been directly demonstrated to be absolutely critical for the biological function of at least a subset of these proteins (36–40). We therefore wished to determine whether the ULD was required for functional activity of IKKβ.

The UBLs of these other proteins do not function as targets for ubiquitination nor are they ligated in a ubiquitin-like manner to separate target proteins (33). Consistent with this, the IKKβ ULD does not contain lysine residues corresponding to Lys48 or Lys63 in ubiquitin that are required for ubiquitin chain formation. The only conserved lysine between the IKKβ ULD and ubiquitin is at position 337 (see asterisk in Fig. 1B); when we mutated this to arginine (K337R), the resultant kinase did not differ from the wild type with respect to basal and induced catalytic activity and the ability to form complexes with IKKa and NEMO (data not shown). Therefore, it appears that like the wider family of UBL domain-containing proteins, the ULD does not function to facilitate IKKβ ubiquitination or ubiquitin-like conjugation with other proteins.

To investigate the function of the ULD, we constructed a deletion mutant of IKKβ lacking the region between residues Leu307 and Met384 (inclusive) which we named IKKβ-d.uld. As shown in Fig. 2A, IKKβ-d.uld failed to activate NF-κB when transiently overexpressed in HeLa cells, whereas similar levels of overexpressed wild-type IKKβ induced robust NF-κB activity in these cells. Furthermore, overexpression of IKKβ-d.uld dose-dependently reduced TNFα-induced NF-κB activity induced in HeLa cells (Fig. 2B). To determine the effects of deleting the ULD on the catalytic function of IKKβ, we overexpressed FLAG-tagged versions of wild-type IKKβ and IKKβ-d.uld in HeLa cells, and then following incubation with TNFα for a range of times up to 120 min, we performed immunoprecipitation kinase assays using GST-IκBα(1–90) as a substrate. As shown in Fig. 2C, catalytic activity of the wild-type kinase was rapidly induced by TNFα, reaching a maximum at 5 min and then returning to basal levels after 30 min (see Fig. 2C, lanes 1–6). Remarkably, and despite being expressed to the same extent as the wild-type kinase, IKKβ-d.uld exhibited no basal or TNFα-induced catalytic activity against IκBα (Fig. 2C, lanes 7–12). We also failed to detect catalytic activation of IKKβ-d.uld in HeLa cells following interleukin-1β treatment (data not shown). Therefore, these findings demonstrate that deletion of the ULD renders IKKβ catalytically inactive against IκBα and refractory to pro-inflammatory cytokine-induced activation.

Several studies (39, 41–43) have established that recombimant versions of only the ubiquitin-like domain of several proteins possess biological activity in cellular overexpression stud-
IKKβ contains a centrally positioned ULD. The domain structure of IKKβ is depicted in A. The relative positions of the kinase domain, leucine zipper (LZ), helix-loop-helix (HLH), and NEMO binding domain (NBD) are shown. The ULD is located between residues Leu307 and Met384. B, sequence alignment of the IKKβ ULD and human ubiquitin. C, alignment of the corresponding region of IKKα with human ubiquitin. B and C, dark and light shading denotes identical and similar amino acids respectively. The asterisk indicates the position of the only conserved lysine residue (Lys337) between ubiquitin and the ULD.

We conclude from these findings that the ULD does not play a role in maintaining the interactions between IKKβ and IKKα or NEMO and is therefore not required for assembly of the “core” IKK complex.

Neither Ubiquitin Nor the Equivalent Region of IKKα Can Functionally Replace the IKKβ ULD—In light of its sequence similarity with ubiquitin, we sought to determine whether the ULD could be functionally replaced within IKKβ by ubiquitin as described previously for the yeast DNA repair protein Rad23 (44). We therefore constructed a panel of deletion and substitution mutants (Fig. 4A), and we noted that each of these kinases interacted with NEMO and each other to the same extent as the wild-type IKK, verifying that these mutations do not affect the inter-molecular interactions within IKK complex (data not shown). We first tested the ability of a FLAG-tagged version of IKKβ in which the ULD was replaced with human ubiquitin (IKKβ-Ub) to activate NF-κB-dependent luciferase activity. As shown in Fig. 4B, neither IKKβ-d.ULD nor the ubiquitin-containing mutant (IKKβ-Ub) could activate NF-κB in a luciferase reporter assay when compared with wild-type IKKβ. Furthermore, similar to IKKβ-d.ULD, IKKβ-Ub was basally catalytically inactive and was not activated following treatment of transfected HeLa cells with TNFα (Fig. 4C, lanes 5 and 6). We next questioned whether the IKKβ ULD sequence could be functionally interchanged with the equivalent region of IKKα, and we constructed an IKKβ mutant containing the 78 residues Ile307 to Val384 of IKKα in place of the ULD (IKKβ-α.78; Fig. 4A). Similar to the ubiquitin substitution mutant, IKKβ-α.78 failed to activate NF-κB-driven luciferase activity (Fig. 4B) and was catalytically inactive and refractory to TNFα-induced activation (Fig. 4C, lanes 7 and 8).
To determine the effects of deleting the corresponding 78 amino acids in IKKβ on its catalytic activity, we constructed a mutant that we named IKKβ-d.78 (Fig. 4A). As shown in Fig. 4D and in contrast to the effects observed with the similar IKKβ mutant lacking the ULD (d.ULD), WT, wild type. Luciferase activity was measured in lysates 48 h after transfection. Expression levels of each construct were determined by immunoblotting (IB) using anti-Xpress (lower panel). B, HeLa cells were transiently transfected with pBIIx-luc together with either vector alone (1st two bars and lanes) or 0.25, 0.5, or 1 μg/ml of Xpress-tagged IKKβ-d.ULD. Forty-eight hours later, cells were either untreated (−) or incubated with TNFα (10 ng/ml; +) for a further 4 h prior to lysis and measurement of luciferase activity. Protein expression levels in lysates were determined using anti-Xpress (lower panel). C, HeLa cells transfected with FLAG-tagged versions of either wild-type IKKβ (lanes 1–6) or IKKβ-d.ULD (lanes 7–12) were treated with 10 ng/ml TNFα for the times indicated. Proteins were immunoprecipitated from lysates using anti-FLAG, and then half of each sample was subjected to kinase assay (KA) using GST-IκBα(1–90) as a substrate (upper panel). The other half of each immunoprecipitate was immunoblotted using anti-FLAG (lower panel). D, HeLa cells were transiently transfected with pBIIx-luc together with either vector alone (1st two bars and lanes) or 0.25, 0.5, or 1 μg/ml of the IKKβ ULD. Forty-eight hours later, cells were either untreated (−) or incubated with TNFα (10 ng/ml; +) for a further 4 h prior to lysis and measurement of luciferase activity.

FIG. 2. The ULD is required for functional activity of IKKβ. A, HeLa cells were transiently transfected with the NF-κB-dependent reporter construct pBIIx-luc together with either vector alone (pcDNA3.1Xpress, Control), Xpress-tagged IKKβ, or increasing amounts (0.25, 0.5, and 1 μg/ml) of an Xpress-tagged IKKβ mutant lacking the ULD (d.ULD). WT, wild type. Luciferase activity was measured in lysates 48 h after transfection. Expression levels of each construct were determined by immunoblotting (IB) using anti-Xpress (lower panel). B, HeLa cells were transiently transfected with pBIIx-luc together with either vector alone (1st two bars and lanes) or 0.25, 0.5, or 1 μg/ml of Xpress-tagged IKKβ-d.ULD. Forty-eight hours later, cells were either untreated (−) or incubated with TNFα (10 ng/ml; +) for a further 4 h prior to lysis and measurement of luciferase activity. Protein expression levels in lysates were determined using anti-Xpress (lower panel). C, HeLa cells transfected with FLAG-tagged versions of either wild-type IKKβ (lanes 1–6) or IKKβ-d.ULD (lanes 7–12) were treated with 10 ng/ml TNFα for the times indicated. Proteins were immunoprecipitated from lysates using anti-FLAG, and then half of each sample was subjected to kinase assay (KA) using GST-IκBα(1–90) as a substrate (upper panel). The other half of each immunoprecipitate was immunoblotted using anti-FLAG (lower panel). D, HeLa cells were transiently transfected with pBIIx-luc together with either vector alone (1st two bars and lanes) or 0.25, 0.5, or 1 μg/ml of the IKKβ ULD. Forty-eight hours later, cells were either untreated (−) or incubated with TNFα (10 ng/ml; +) for a further 4 h prior to lysis and measurement of luciferase activity.

To determine the effects of deleting the corresponding 78 amino acids in IKKα on its catalytic activity, we constructed a mutant that we named IKKα-d.78 (Fig. 4A). As shown in Fig. 4D and in contrast to the effects observed with the similar IKKβ mutant, deletion of this entire region had no effect on the ability of FLAG-IKKα to phosphorylate IκBα in response to TNFα. However, despite its ability to phosphorylate IκBα in vitro, the major function of IKKα in NF-κB activation is as the critical kinase necessary for phosphorylating the NF-κB2 precursor protein p100 in the noncanonical NF-κB pathway (24–26). Activation of IKKα in this pathway is dependent upon NIK and results in phosphorylation-induced proteolytic processing of p100 to p52 (24–26). We therefore tested the effects of deleting the ULD-corresponding region of IKKβ on its catalytic activity and as demonstrated in Fig. 4E, IKKα-d.78 remained capable of mediating NIK-induced p100 processing to p52. Hence this region appears to be dispensable for the known physiological functions of IKKα.

We conclude from these experiments that an intact ULD is exquisitely required for IKKβ activity and cannot be functionally substituted with ubiquitin or the corresponding region of IKKβ. In contrast, catalytic activity of IKKα against both IκBα and p100 does not appear to require the presence of the equivalent region of that kinase.
Mutational Analysis of the IKKβ ULD—It is possible that the effects of deleting the entire ULD on IKKβ activity might be due to gross structural disruption resulting from loss of a relatively large portion of the kinase. To address this issue we constructed a panel of five smaller subdomain deletion mutants lacking stretches of between 11 and 17 residues within the ULD. The positions of the subdomains that we named regions A to E are illustrated in Fig. 5A. As shown in Fig. 5B, versions of IKKβ sequentially lacking regions A, B, C, or D failed to activate NF-κB in a luciferase reporter assay. In contrast, the mutant lacking region E of the ULD (Del.E) induced NF-κB activity to a similar level as the wild-type kinase (Fig. 5B). Consistent with these data, we found that only the IKKβ-Del.E mutant displayed TNFα-induced catalytic activity against GST-IκBα, which resembled the activity of wild-type IKKβ (Fig. 5C, lanes 11 and 12). Moreover, although we observed low levels of catalytic activity with each of the other deletion mutants, only IKKβ-Del.D consistently exhibited detectable inducibility in response to TNFα stimulation, although the magnitude of this activity was significantly less than either wild-type IKKβ or IKKβ-Del.E (Fig. 5C, compare lanes 9 and 10 with lanes 1, 2, 11, and 12). These findings strongly suggest that maintenance of the overall integrity of the region between Leu311 and Ala367 of the ULD corresponding to the subdomains designated A-D in Fig. 5A is absolutely critical for the functional activity of IKKβ.

In a further attempt to identify any potentially critical functional sites within the IKKβ ULD, we performed sequence alignment analysis to determine whether the domain contained any residues at positions conserved among the family of UBL-containing proteins (33). As shown in Fig. 6A, alignment of the IKKβ ULD (Leu311–Met364) with the UBLs of Bag-1, BAT-3, OASL, HHR23A, HHR23B, as well as human ubiquitin identified a cluster of five residues that were conserved among all of the proteins. These residues in IKKβ were specifically proline at position 347 (Pro347), glutamate at 351 (Gln351), leucine at 353 (Leu353), glycine at 358 (Gly358), and leucine at position 361 (Leu361). When we extended the sequence alignment to over 20 distinct UBL-containing proteins from species ranging from yeast to human, these same residues were conserved among all proteins analyzed (data not shown). We therefore surmised that the residues at these positions might play an important role in the function of this domain, and to test this we constructed a panel of single point mutants in which each residue was substituted with alanine. Consistent with our previous observations (Fig. 3), all of these IKKβ point mutants interacted with NEMO and IKKα (data not shown).

To test the effects of these alanine substitutions on the ability of IKKβ to induce transcriptionally active NF-κB, we performed a luciferase reporter assay, and as shown in Fig. 6B, the P347A and L353A mutants induced NF-κB activity to the same level as wild-type IKKβ. Similarly, although the Q351A mutant tended to be less active, over the course of multiple experiments, its ability to induce NF-κB activity did not significantly vary from that of the wild-type kinase (not shown). In contrast, NF-κB activity induced by G358A was consistently reduced when compared with wild-type IKKβ, and more strikingly, the L353A mutant did not induce activity above the basal levels observed in vector-alone transfected control cells (Fig. 6B). We were therefore surprised to find that despite its inability to activate NF-κB, L353A exhibited TNFα-induced catalytic activity against GST-IκBα (Fig. 6C). Similar catalytic activity was also observed for all of the other alanine mutants including G358A (data not shown). The failure of IKKβ-L353A to activate NF-κB (Fig. 6D) led us to question whether phosphorylation by the mutant kinase could lead to IκBα degradation. We therefore transfected HEK293 cells with wild-type IKKβ, IKKβ-L353A, or IKKβ-D-ULD, and we determined the effects on both basal and TNFα-induced levels of IκBα. As shown in Fig. 6D, consistent with our in vitro kinase assay, transfection of HEK293 cells with both the wild-type and L353A mutant kinases decreased the amount of basal IκBα in cells (Fig. 6D, compare lanes 1, 5, and 13). In contrast, IκBα levels in IKKβ-D-ULD transfected were unchanged compared with control (Fig. 6D, lanes 9 and 13). Furthermore, IκBα was degraded following TNFα treatment in wild-type and L353A-transfected cells with similar kinetics as control cells, whereas in d.ULD-transfected cells, IκBα degradation was impaired (Fig. 6D, compare lanes 12 and 16). This is consistent with the lack of catalytic activity we observed for IKKβ-D-ULD (Fig. 2C). Taken together, these findings demonstrate that although IKKβ-L353A is capable of phosphorylating IκBα and causing its degradation...
degradation in response to TNFα, this single point mutation prevents the overexpressed kinase from activating transcriptionally competent NF-κB.

**IKKβ-L353A and IKKβ-d.ULD but Not Wild-type IKKβ Form a Complex with the NF-κB p65 Subunit**—Previous studies (45, 46) have demonstrated that IKKβ can phosphorylate the serine residue at position 536 within the C terminus of the NF-κB p65 subunit and that this phosphorylation is critical for its transcriptional activity. We therefore surmised that IKKβ-L353A might be unable to phosphorylate Ser536 in p65, thereby accounting for its failure to induce transcriptionally active NF-κB (Fig. 6B) despite its ability to phosphorylate IκBα (Fig. 6C) and cause its degradation (Fig. 6D). To test this hypothesis, we transfected HeLa cells with either p65 alone or p65 in the presence of wild-type IKKβ, IKKβ-L353A, or dominant negative IKKβ (K44M), and we performed a luciferase reporter assay. Consistent with our hypothesis, we found that wild-type IKKβ enhanced p65-induced luciferase activity, whereas transcriptional activity of p65 that was co-transfected with either K44M or L353A was severely impaired (Fig. 7A). We next performed immunoprecipitation kinase assays using either the

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

**FIG. 4. The IKKβ ULD cannot be functionally substituted with ubiquitin or the equivalent region of IKKα.** A, the structures of wild-type IKKβ and IKKα and the various deletion and substitution mutants are shown. B, HeLa cells were transiently transfected with pBIIx-luc together with either vector alone (pFLAG-CMV2: Control), FLAG-tagged IKKβ (WT), or the mutants indicated, and luciferase activity was measured in lysates 48 h after transfection. Expression levels of each construct were determined by immunoblotting (IB) using anti-FLAG (lower panel). C, HeLa cells were transiently transfected with the FLAG-IKKβ constructs indicated, and after 48 h, cells were either untreated (−) or treated (+) for 5 min with 10 ng/ml TNFα. Proteins were recovered from lysates using anti-FLAG and an immune complex kinase assay was performed using GST-IκBα-(1–90) as a substrate (lanes 1–8). Proteins in identical lysates from simultaneously transfected cells were immunoprecipitated and immunoblotted using anti-FLAG (lanes 9–12). D, HeLa cells were transiently transfected with the FLAG-IKKα constructs indicated and then processed for kinase assay (lanes 1–4) and immunoblotting (lanes 5 and 6) as described in C. E, HEK293 cells were transiently transfected with pBIIx-luc (Control; lanes 1 and 2), FLAG-IKKβ (WT; lanes 3 and 4), or FLAG-IKKα (d.78; lanes 5 and 6) either alone (−) or together with NIK (+), and then resulting lysates were sequentially immunoblotted with anti-p100 (upper panel) and anti-FLAG (lower panel). The positions of p100, p52, and a nonspecific band (n.s) are indicated.
with both the L353A and d.ULD IKKβ mutants, whereas it was not pulled down with the wild-type kinase. These results were recapitulated when we transfected HeLa cells with IKKβ and the ULD mutants and tested their ability to interact with endogenous p65. Thus, FLAG-tagged IKKβ-L353A and IKKβ-d.ULD associated with endogenous p65 (Fig. 7D, lanes 3 and 4) although no such interaction with the wild-type kinase could be detected (lane 2). These findings therefore suggest that the failure of L353A to induce transcriptional activity of NF-κB is not due to a lack of catalytic activity against either IκBα or the C terminus of p65 but may instead be related to its ability to physically interact with p65.

DISCUSSION

In this study we sought to identify regions of IKKα or IKKβ that are unique for either kinase. By using a series of bioinformatic strategies, we identified a novel ubiquitin-like domain within IKKβ that was not detected in IKKα. Deletion and small mutations within the ULD profoundly affected IKKβ function, demonstrating that it is a critical regulatory domain required for activity of the kinase. Furthermore, our domain-swap analysis (Fig. 4) strongly suggests that the domain is absolutely specific for IKKβ and dispensable for catalytic function of IKKα. In this regard versions of IKKβ that either lacked the entire ULD or had this region replaced with either ubiquitin or the corresponding region of IKKα were completely catalytically inactive against IκBα and were unable to induce NF-κB-dependent gene expression. This is entirely consistent with a previous report (47) in which the catalytic domain of IKKβ (residues 1–301) was fused with the C terminus of IKKα (residues 301–745), resulting in catalytic inactivity of that chimera against IκBα. It therefore appears that an intact ULD is absolutely critical for IKKβ to phosphorylate IκBα. In contrast, loss of the ULD-corresponding region of IKKα did not affect its ability to both phosphorylate IκBα and induce p100 processing in response to NIK. Therefore, we hypothesize that the presence of the ULD in IKKβ contributes significantly to the functional divergence of the IKKs. In this regard, it is possible that the presence of this domain, resembling the highly evolutionarily conserved ubiquitin protein, underlies the more ancient innate immune and inflammatory functions mediated by IKKβ via the classical NF-κB pathway (17). It is tempting to speculate further that the role of IKKα in mediating critical aspects of the adaptive immune response (i.e. lymphoid organogenesis and B-cell maturation) may be a result of evolutionary modification within the ULD, resulting in the functional divergence of the kinases. Clearly, however, a full understanding of the precise function of the ULD will be required before any conclusions can be drawn concerning its role in shaping the distinct biological functions of the IKKs.

We have demonstrated that the ULD is not involved in formation of the core IKK complex composed of IKKα, IKKβ, and NEMO. Thus we found that deletion of the ULD does not affect the ability of IKKβ to heterodimerize with IKKα. Furthermore, consistent with our prior identification of the NBD in the extreme C terminus of both IKKs (30, 31), deletion of the ULD did not affect the interaction of IKKβ with NEMO. Previous workers (48) have suggested that in addition to the NBD, a separate interaction domain for NEMO exists within IKKβ. Our data demonstrate that if such a region exists, it is not the ULD as the interaction with NEMO was clearly unaffected following its deletion. We also found that insertion of either ubiquitin or the corresponding region of IKKα into IKKβ, or deletion of the equivalent IKKα residues or insertion of the ULD into IKKα did not affect IKK heterodimerization or the ability of either IKKβ or IKKα to interact with NEMO (data not shown). These findings therefore lead us to conclude that the ULD is not required for the maintenance of IKK complex architecture. It remains an intriguing possibility that the ULD functions as a protein–protein interaction domain that facilitates the association with unknown IKKβ-specific interacting proteins (see below).
We considered the possibility that the ULD might play a role in ubiquitination of IKKβ or might be required to physically conjugate IKKβ with unknown target proteins. However, we have failed to detect such modifications throughout the course of our experiments. Furthermore, two lines of evidence suggest that the ULD does not play a role in facilitating ubiquitination or ubiquitin-mediated conjugation of IKKβ. First, we initially identified the ULD by using the PROSITE data base as having similarity with the type 2 UBL (UBIQUITIN_2) profile present in a large family of proteins (33). To date, however, no evidence exists to support a role for this type of domain in ubiquitination nor have such domains been reported to conjugate with target proteins. In contrast, the type 2 domain defines a family of proteins in which the UBL functions as a linear noncleavable insertion that appears to have a primary role in maintaining specific protein-protein interactions (33). The second piece of evidence against the role in ubiquitination is that the IKKβ ULD does not contain lysines in either of the positions that correspond to Lys48 and Lys63 in ubiquitin. Lysines at these positions are required for ubiquitin conjugation and chain formation, yet the only lysine that is conserved between ubiquitin and the ULD is Lys337 of IKKβ (Fig. 1B). The corresponding lysine in ubiquitin (Lys27) is not a target for ubiquitin chain formation. Nevertheless, to test the potential importance of this residue, we have constructed a lysine to arginine substitution mutant (K337R) of IKKβ, and we found that this mutation did not affect the basal or induced catalytic function or the complex forming capability of IKKβ (not shown). We therefore believe that the ULD is not a target for IKKβ ubiquitination or ubiquitin-like conjugation of IKKβ with other proteins.
as molecular chaperones that present ubiquitinated cargo proteins to proteasomal ATPases where they are subsequently unfolded and degraded, although leaving the UBL protein intact (33, 36–40, 42, 43, 49–53).

This function of certain UBL-containing proteins presented a fascinating hypothesis regarding the potential role of the ULD in IKKβ. Thus we considered that the ULD might facilitate an interaction between the IKK complex and proteasome thereby bringing the kinase, its ubiquitinated substrate (i.e. IkB proteins), and the degradation machinery into close context. Furthermore, association with the proteasome may explain our failure to detect overexpressed ULD alone as it might be rapidly degraded via this route. Nevertheless, despite intense effort using a wide range of available reagents, we have been unable to detect any interactions between either endogenous or overexpressed IKKβ and the proteasome. It therefore appears that the IKKβ ULD does not function in manner similar to the UBLs of HHR23A and -B, PLIC-1 and -2, Parkin, or Bag-1. This finding is perhaps not completely surprising as the UBL is located in the extreme N terminus of all of the UBL-containing proteins that interact with the proteasome, whereas the ULD is centrally positioned in IKKβ. Furthermore, the proteasome-interacting proteins also contain ubiquitin-associated domains through which they can interact with ubiquitinated proteins. In addition, none of these proteins are kinases and appear to function primarily as molecular adaptors or chaperones. Finally, our domain swap analysis demonstrated that the function of the ULD could not be replaced with ubiquitin, whereas insertion of ubiquitin into the UBL site in Rad23 has been reported to maintain the function of that protein (44). Thus, it appears that the proteasome-binding capability of ubiquitin is insufficient to maintain the function of IKKβ. It is therefore likely that the ULD represents a distinct type of ubiquitin-like domain that performs a function separate from proteasomal localization.

The possibility therefore remains that the ULD plays a role in maintaining interactions with separate IKKβ-specific proteins. One particular set of candidates for such interacting proteins may be components of the COP9 signalsome that is a distinct protein complex that exhibits similarities to the 26S proteasome (54). Most intriguingly, catalytic activity specific for IkBo has been found associated with the COP9 signalsome,

\[ \text{IkBo} \]

2 M. J. May and S. Ghosh, unpublished observations.
and components of COP9 have been shown to interact with the classical IKK complex (54, 55). We are currently investigating the potential role of the ULD in mediating interaction of IKKβ with specific proteins within the COP9 signalosome.

Although the potential of the ULD to operate as an interaction domain remains to be elucidated, our findings are consistent with such a function being important for IKK activity and NF-κB activation. For example, overexpression of just the ULD blocked TNFα-induced NF-κB activation, and it is possible that this disrupted or prevented a critical IKKβ-specific interaction. Structural studies of the interaction of UBL-containing proteins with the ubiquitin-interacting motifs within proteasomal subunits have demonstrated that the binding face of the UBL is composed of distinct hydrophobic residues spread throughout the length of the UBL (36). For example, in the case of HHR23A interacting with the second ubiquitin-interacting motif of the proteasomal S5a subunit, critical UBL residues in HHR23A are located on distinct and separated β-sheets (i.e. Leu10, Lys47, Leu48, Ile49, Lys51, Ile54, Leu55, Val73, and Met75 of HHR23B). This requirement for multiple residues contributing to the binding face in these proteins is consistent with the results of our subdomain deletion analysis of the IKKβ ULD. We found that deletion of the first four smaller regions within the ULD (regions A–D in Fig. 5A) but not the most C-terminal 17 amino acids (region E in Fig. 5A) rendered IKKβ catalytically inactive. Thus, if the ULD is involved in protein-protein interactions, the binding face that facilitates this association is most likely composed of residues in each of the subdomains A–D. Nevertheless, positive identification of a bona fide ULD-binding protein and subsequent in-depth structural analysis will be required to determine the full range of critical residues within the ULD.

Most intriguingly, we found that a cluster of five amino acids in the IKKβ ULD was conserved among many UBL-containing proteins. Of these residues, we demonstrated through alanine-scanning mutation that Leu353 is absolutely critical for the ability of IKKβ to activate NF-κB. Close examination of the residues in HHR23 proteins, Parkin, and hPLIC2 that facilitate their interaction with the proteasome clearly defines a common β-sheet that encompasses this corresponding leucine residue in these proteins as being absolutely critical for maintaining the interaction (36, 37, 40). For each of these proteins, however, the crucial residue appears to be an isoleucine immediately after the leucine (i.e. Ile49 in HHR23A) (36), and in IKKβ this residue is also a leucine (Leu354). It is therefore possible that the inability of IKKβ to interact with the proteasome may due at least in part to this subtle difference in this specific subregion of the ULD and that this together with as yet unknown separate binding residues may impart binding specificity for novel interacting partners. Identification of Leu353 in IKKβ as a critical residue that occupies a position required for distinct UBL-protein function therefore lends strong support to the hypothesis that the ULD facilitates protein-protein interactions.

Although our studies have not definitively determined the function of the ULD, we consistently found that both the ULD deletion and L353A mutants of IKKβ formed a stable complex with p65 that was not observed with the wild-type kinase. Thus, we were able to immunoprecipitate both co-expressed and endogenous p65 with FLAG-tagged versions of both mutants but not with wild-type IKKβ (Fig. 7). We do not yet know whether this interaction in itself is sufficient to prevent NF-κB activation, but our observation that IKKβ-L353A induces IκBα degradation but does not activate NF-κB-dependent gene expression (i.e. luciferase activity) strongly suggests that this is the case. It is therefore possible that the IKKβ ULD mutants sequester p65 in the cytoplasm; however, our preliminary experiments suggest that this is not the case (data not shown). Alternative explanations may be that the associated IKKβ mutants prevent the association of p65 with its target DNA in gene promoters or prevent the interaction of critical transcriptional cofactors such as the CREB-binding protein (32). Further experiments will clearly be required to determine which if any of these mechanisms account for the failure of IKK ULD mutants to activate NF-κB-dependent gene expression.

Previous studies have demonstrated that IKKβ preferentially phosphorylates IκBα bound to p65:p50 (19), and p65 was found associated with the purified IKK complex when IKKβ was first described (5). Together these findings therefore suggest that IKKβ interacts with p65 either directly or indirectly while phosphorylating the specific N-terminal serine residues of IκBα. Our finding that the ULD deletion mutant associates with p65 suggests that the domain is not required to facilitate this interaction but is in fact necessary for the disassociation of IKKβ from p65. Most interestingly, the single point mutation at Leu353 was also sufficient to cause an interaction with p65, suggesting that this is indeed a highly critical residue for this function of the ULD. It is therefore possible that the ULD recruits a novel protein that is involved in disassembling the IKKβ-NF-κB complex following IκBα phosphorylation. Alternatively, a conformational change that requires a functional ULD may be required for p65 release, and mutation within the ULD might prevent this from occurring. It is interesting to note that the ULD deletion mutant was unable to phosphorylate IκBα, whereas the L353A mutant could do so. This suggests that in addition to regulating the interaction with p65 that the ULD might also play a role in coordinating the recognition of the substrate. In depth structural analysis of the IKK-p65-IκB complex will help identify the role of the ULD in regulating these interactions within the IKK-NF-κB supercomplex.

In conclusion, this study demonstrates the existence of a novel domain in IKKβ that is absolutely critical for its functional activity. The ULD is not present in IKKα, and deletion of the equivalent region in that kinase does not affect its function. We therefore hypothesize that the ULD contributes to the functional divergence known to exist between these kinases. Although further work will be required to fully establish the precise function of the ULD in IKKβ, our findings suggest that it plays a role in the detachment of IKKβ from p65 following IκBα phosphorylation. The ULD therefore provides a promising target for drugs aimed at specifically blocking the activity of IKKβ and the classical NF-κB pathway while keeping IKKα function and noncanonical signaling intact.

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A Novel Ubiquitin-like Domain in IκB Kinase β Is Required for Functional Activity of the Kinase
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