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Mapping of the Inducible IκB Phosphorylation Sites That Signal Its Ubiquitination and Degradation†

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Extracellular stimuli that activate the transcription factor NF-κB cause rapid phosphorylation of the IκBα inhibitor, which retains NF-κB in the cytoplasm of nonstimulated cells. Phosphorylation of IκBα is followed by its rapid degradation, the inhibition of which prevents NF-κB activation. To determine the relationship between these events, we mapped the inducible phosphorylation sites of IκBα. We found that two residues, serines 32 and 36, were phosphorylated in response to either tumor necrosis factor, interleukin-1, or phorbol ester. Substitution of either serine blocks or slows down induction of IκBα degradation. Substitutions of the homologous sites in IκBβ, serines 19 and 23, also prevent inducible IκBβ degradation. We suggest that activation of a single IκB kinase or closely related IκB kinases is the first critical step in NF-κB activation. Once phosphorylated, IκB is ubiquitinated. Unlike wild-type IκBα, the phosphorylation-defective mutants do not undergo inducible polyubiquitination. As substitution of a conserved lysine residue slows down the ubiquitination and degradation of IκBα without affecting its phosphorylation, polyubiquitination is required for inducible IκB degradation.

The transcription factor NF-κB is composed of heterodimeric and homodimeric complexes of the Rel proteins p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), RelB, and c-Rel (reviewed in references 7, 22, 33, and 42). Originally described as a constitutively active nuclear factor in B lymphocytes (41), NF-κB is cytoplasmic in most other cell types, but following exposure to a variety of extracellular stimuli, it translocates to the nucleus and activates transcription of specific target genes (reviewed in references 4, 23, and 42). The cytoplasmic retention of NF-κB in nonstimulated cells is mediated by a family of inhibitor proteins, the IκBs (reviewed in references 5 and 20). As specific molecular reagents were first available for IκBα, more is known about its regulation than about other IκBs. Exposure of cells to NF-κB-activating stimuli, including tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), lipopolysaccharide (LPS), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and UV irradiation, results in rapid IκBα degradation (6, 11, 14, 15, 27, 34). Recently some of these stimuli were also shown to trigger degradation of IκBβ, albeit with kinetics different from that for IκBα degradation (44). In addition, inhibitors of IκBα degradation, most notably peptide aldehydes that also inhibit the multifunctional protease (26S proteasome), were found to prevent NF-κB activation (2, 16, 17, 32, 38, 39, 46). It was therefore suggested that degradation of IκB by the proteasome is an essential early step in the NF-κB activation pathway (reviewed in references 13 and 43).

Degradation of IκBα is preceded by its phosphorylation. Although phosphorylation of IκBα does not cause its dissociation from NF-κB, it appears that the phosphorylated form may be preferentially degraded (2, 16, 17, 32, 38, 46). Phosphopeptide mapping suggested that inducible IκBα phosphorylation occurs on a small number of sites (16). Recently, through the use of site-directed mutagenesis, it was suggested that these sites include S-32 and S-36 (9, 10, 45, 50). However, no biochemical proof that S-32 and S-36 are indeed the inducible phosphoacceptor sites was provided, and it remains possible that substitution of these serines affects IκBα phosphorylation or metabolism indirectly. To gain better insight to the mechanism of NF-κB activation and the nature of the IκB kinase(s), we undertook biochemical identification of the inducible phosphorylation sites on IκBα. Using phosphopeptide mapping and phosphoamino acid analysis coupled to digestion with different proteases, we identified the inducible phosphoacceptor sites as S-32 and S-36. This assignment was confirmed by site-directed mutagenesis, and the resulting mutants were stable following cell stimulation with either TNF-α, IL-1, or TPA. Interestingly, replacement of S-32 and S-36 by threonines, a substitution that usually does not interfere with protein phosphorylation by serine/threonine kinases, slowed down or blocked IκBα phosphorylation and degradation. Substitutions for these serines also interfered with conversion of IκBα to higher-molecular-weight polyubiquitinated forms. Substitutions at the homologous sites in IκBβ, S-19 and S-23, had a similar effect and blocked signal-induced IκBβ degradation. These results strongly suggest that both IκBs are targeted by the same kinases or a small group of similar kinases and are not regulated by separate pathways as previously suggested. We also identified a conserved lysine residue whose substitution interfered with the inducible polyubiquitination and degradation of IκBα but not with its phosphorylation.

MATERIALS AND METHODS

Plasmids. pHA-MAD-3 is a 3× hemagglutinin (HA)-tagged (51) version of MAD-3 (IκBα) containing the *Nco*I-to-*Not*I insert from pKGMAD-3 (16) inserted in frame behind the HA tag in pBluescript (KS$^+$) (Stratagene). The different substitution mutants were made with the appropriate high-pressure

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† Dedicated to the memory of our friend Yoav Citri.

liquid chromatography-purified oligonucleotides (sequences available upon request) by using a Chameleon double-stranded DNA mutagenesis kit (Stratagene) with pHA-MAD-3 as the template. Mutants were sequenced on an ABI PRISM DNA sequencing system. pHA-IκBβ was made by inserting an *Nco*I-to-*Not*I insert from pBS-15f (44) in frame behind the HA tag in pBluescript (KS^+) and was used as a template for making the different IκBβ substitution mutants by the methods described above. The wild-type (WT) and mutant HA-tagged IκBs were excised from the pBluescript backbone and cloned between the *Hin*dIII and *Not*I sites of the pRc/β actin expression vector (26).

Antibodies and peptide aldehydes. Anti-IκBα, anti-p65-C, and anti-IκBβ antibodies were described previously (15, 16, 44). Anti-p65 carboxyl-terminal antibody 519 (17) was a generous gift from A. Baldwin. The antiubiquitin antibody was raised against sodium dodecyl sulfate (SDS)-denatured ubiquitin (24) and was a generous gift from A. Haas. Anti-HA monoclonal antibody 12CA5 (51) was purified from ascites fluid. Affinity-purified antibodies against IκBα (c-21) and IκBβ (sc-20) were purchased from Santa Cruz Biotechnology. *N*-Acetyl-Leu-Leu-norleucinal (Ac-LLnL-CHO) was purchased from Calbiochem (San Diego, Calif.). Benzyloxycarbonyl-Leu-Leu-phenylalaninal (Z-LLF-CHO) (49) was a generous gift of M. Orlowski and from Signal Pharmaceuticals. All peptide aldehydes were dissolved in absolute ethanol and stored at −80°C.

Cells and transfections. NIH 3T3 and HeLa S3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 2 mM glutamine. Stably transfected HeLa cell pools were made by transfecting 3×10^5 HeLa cells with either WT or mutant HA-IκB expression vectors (1.5 μg) and Lipofectamine (6 μl) as suggested by the manufacturer (Gibco-BRL). After 24 h, the medium was changed and G418 (1 mg/ml; Gibco-BRL) was added. Individual clones (at least 30 to 40 per plate) were pooled and propagated in the presence of G418.

Cell extracts. Nuclear extracts were prepared and mobility shift assays were performed as previously described (15). Cell-free translated proteins were prepared as previously described (36). To analyze IκB degradation, cells were either left untreated or stimulated with TNF-α (10 ng/ml), IL-1α (2 ng/ml; R&D Systems) or TPA (100 ng/ml; Sigma) for various times before being harvested in ice-cold phosphate-buffered saline (PBS) and pelleted at 2,000 × *g* at room temperature for 30 s. The cells were resuspended on ice in whole cell extract lysis buffer containing 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.7), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5% (vol/vol) Triton X-100, 3 mM dithiothreitol, 40 mM β-glycerophosphate, 50 mM NaF, 1 mM Na_3VO_4, 20 mM *p*-nitrophenyl phosphate (Calbiochem), 10 μg each of aprotinin, leupeptin, bestatin, and pepstatin (all from Calbiochem) per ml, 100 μM tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK), and 1 mM phenylmethylsulfonyl fluoride (both from Sigma). Lysates were rotated for 45 min at 4°C and centrifuged at 13,000 × *g* for 15 min. Protein concentration of the extracts was determined by the method of Bradford with a Bio-Rad dye system. SDS sample buffer was added to the supernatants, which were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to Immobilon-P membranes. Immunoblot analysis was performed essentially as described previously (16) except that SDS–12% polyacrylamide gels were used.

Immunoprecipitation and peptide mapping. Immunoprecipitation of IκBα and p65 was performed as described previously (16, 35, 36). Cells were metabolically labeled with $^{32}P_i$ (NEN-DuPont) as described previously (16). After 4 h of labeling, the cells were stimulated with TNF-α (10 ng/ml), IL-1 (2 ng/ml), or TPA (100 ng/ml) and harvested in cold radioimmunoprecipitation assay (RIPA) buffer supplemented with phosphatase and protease inhibitors (16). When used, proteasome inhibitors were added 1.5 h prior to harvesting. IκBα was immunoprecipitated with IκBα antibody (16); HA-IκBβ was immunoprecipitated with anti-HA antibody 12CA5. After fractionation of the immune precipitates by SDS-PAGE (12% polyacrylamide gels) and transfer to nitrocellulose (Schleicher & Schuell), the phosphorylated IκB bands were identified by autoradiography, excised, and digested with various proteases (trypsin [Worthington]; V8, LYS-C, ASP-N, or thermolysin [Calbiochem]) or CNBr (Sigma). The resulting peptides were analyzed either by Tris-Tricine gel electrophoresis (TTE) (40) or by two-dimensional phosphopeptide mapping (8, 16). For TTE analysis, the dried peptides were resuspended in 1× TTE sample buffer (0.0625 M Tris-Cl [pH 6.8], 1% SDS, 2.5% β-mercaptoethanol, 10% glycerol, 0.000125% bromophenol blue), boiled for 5 min, and fractionated on a Tris-Tricine gel consisting of a 24.75% polyacrylamide resolving gel (15 cm) containing 15% glycerol overlaid by a 10% polyacrylamide spacer gel (2 to 3 cm) and a 4% stacking gel. The spacer and resolving gel buffer was 0.1 M Tris-Cl (pH 8.45) and 0.1% SDS. The anode buffer was 0.2 M Tris-Cl (pH 8.9), and the cathode buffer (pH ~8.25) was 0.1 M Tris, 0.1 M Tricine, and 0.1% SDS. Electrophoresis was at 30 V until the samples had migrated into the stacking gel, at which time the voltage was increased to 100 V. After running, the gel was fixed for 45 min in 45% methanol–10% acetic acid–15% glycerol and then dried and exposed to X-ray film at −80°C with an intensifying screen. Molecular mass standards (31 to 2.5 kDa; Promega), bacitracin (1.41 kDa), aprotinin (6.52 kDa), elastase substrate I (0.59 kDa), and [Leu^5]-enkephalin (0.56 kDa; Calbiochem) were used to calibrate the gels.

Analysis of IκB ubiquitination. Cells were either left untreated or treated with the proteasome inhibitor Z-LLF-CHO (10 μM) for 60 min and then incubated in the absence or presence of TNF-α (10 ng/ml) or IL-1 (2 ng/ml). At the indicated times, the cells were harvested in ice-cold PBS and lysed on ice in cold RIPA buffer supplemented with protease and phosphatase inhibitors as described above, 10 mM *N*-ethylmaleimide (NEM; Calbiochem), and 0.2% SDS. The lysates were centrifuged at 13,000 × *g* for 25 min at 4°C. SDS sample buffer was added to the supernatants, which were then boiled for 10 min and fractionated on SDS–10% polyacrylamide gels. The proteins were transferred to Immobilon-P membranes (Millipore), and the immunoblots were probed with affinity-purified IκBα or IκBβ antibody (Santa Cruz Biotechnology) or partially purified monoclonal antibody 12CA5 (anti-HA). Similar procedures were used to analyze anti-HA immunoprecipitates except that the immune complexes (isolated as described previously [36]) were washed in NEM-supplemented RIPA buffer and eluted with 0.1 M CAPSO (3-[cyclohexylamino]-2-hydroxy-1-propanesulfonic acid; pH 11.25; Sigma). The eluate was fractionated by SDS-PAGE (10% polyacrylamide gel) and transferred to an Immobilon-P membrane. Immunoblot analysis included affinity-purified antiubiquitin (conjugate-selective) antibody (23). To detect IκBα forms associated with p65 (RelA), cells were pretreated with Z-LLF-CHO as described above and then left unstimulated or stimulated with TNF-α (10 ng/ml) for 12 min. The cells were then harvested and lysed in buffer which preserves the p65-IκBα complex (16). The lysis buffer was also supplemented with 10 mM NEM. The lysates were immunoprecipitated with C-terminal p65 antibody 519 or its preimmune serum. Immunoprecipitates were fractionated and analyzed by immunoblot analysis with IκBα antibody c-21 as described above.

RESULTS

Biochemical identification of the inducible phosphorylation sites in IκBα. IκBα is phosphorylated to a low extent in resting cells, and upon cell stimulation with either TNF-α, IL-1, or TPA, it is highly phosphorylated on several new sites (16). To identify the inducible IκBα phosphorylation sites, we used high-resolution TTE to fractionate proteolyzed ^{32}P-labeled IκBα isolated from nonstimulated and TNF-α-stimulated HeLa or NIH 3T3 cells. CNBr cleavage yielded a phosphopeptide of approximately 2.5 kDa that contained the inducible phosphorylation sites, along with a few other larger peptides (which are partial cleavage products [data not shown]). Since CNBr cleaves at methionine residues, the only way it could generate a 2.5-kDa fragment of IκBα is by cleavage at M-13 and M-37 (Fig. 1A). Digestion of human IκBα with V8 protease generated a 2-kDa peptide containing the inducible phosphorylation sites (Fig. 1A). This phosphopeptide is most likely generated by cleavage at E-23 and E-40, as no other V8 digestion sites could give rise to a phosphopeptide of a similar size. Digestion of both human and mouse IκBαs with endopeptidase LYS-C, which cleaves after lysine residues, produced a 4-kDa phosphopeptide containing the inducible phosphorylation sites. Considering the CNBr and V8 protease results and the distribution of lysines in the N-terminal portion of IκBα, this peptide is most likely due to a partial digest. As only the N-terminal portions of human and mouse IκBαs contain properly spaced lysines that can give rise to such a peptide, these results are consistent with those of the CNBr and V8 digests.

Digestion with trypsin, which cleaves after positively charged residues, produced three phosphopeptides of approximately 1.0, 1.8, and 2.4 kDa that contain the inducible phosphorylation sites of both human and mouse IκBαs. A larger, 6-kDa peptide contains the constitutive phosphorylation sites (Fig. 1A). Two-dimensional analysis of tryptic digests of human and mouse IκBαs from TNF-α-stimulated cells revealed the presence of three major phosphopeptides (Fig. 1B) that, on the basis of comparison with IκBαs isolated from nonstimulated cells, reflect inducible phosphorylation (data not shown, but see reference 16). Two-dimensional separation of a mixture of trypsin-digested human and mouse IκBαs revealed that two of the phosphopeptides (1 and 3) comigrated and therefore are likely to be identical. Phosphopeptide 2, however, exhibited species-specific differences in its mobility, with the human version being more hydrophobic (Fig. 1B). The inducible tryptic phosphopeptides of human IκBα were isolated after two-dimensional separation and subjected to a secondary digest with endopeptidase ASP-N, which cleaves amino terminal to aspar-

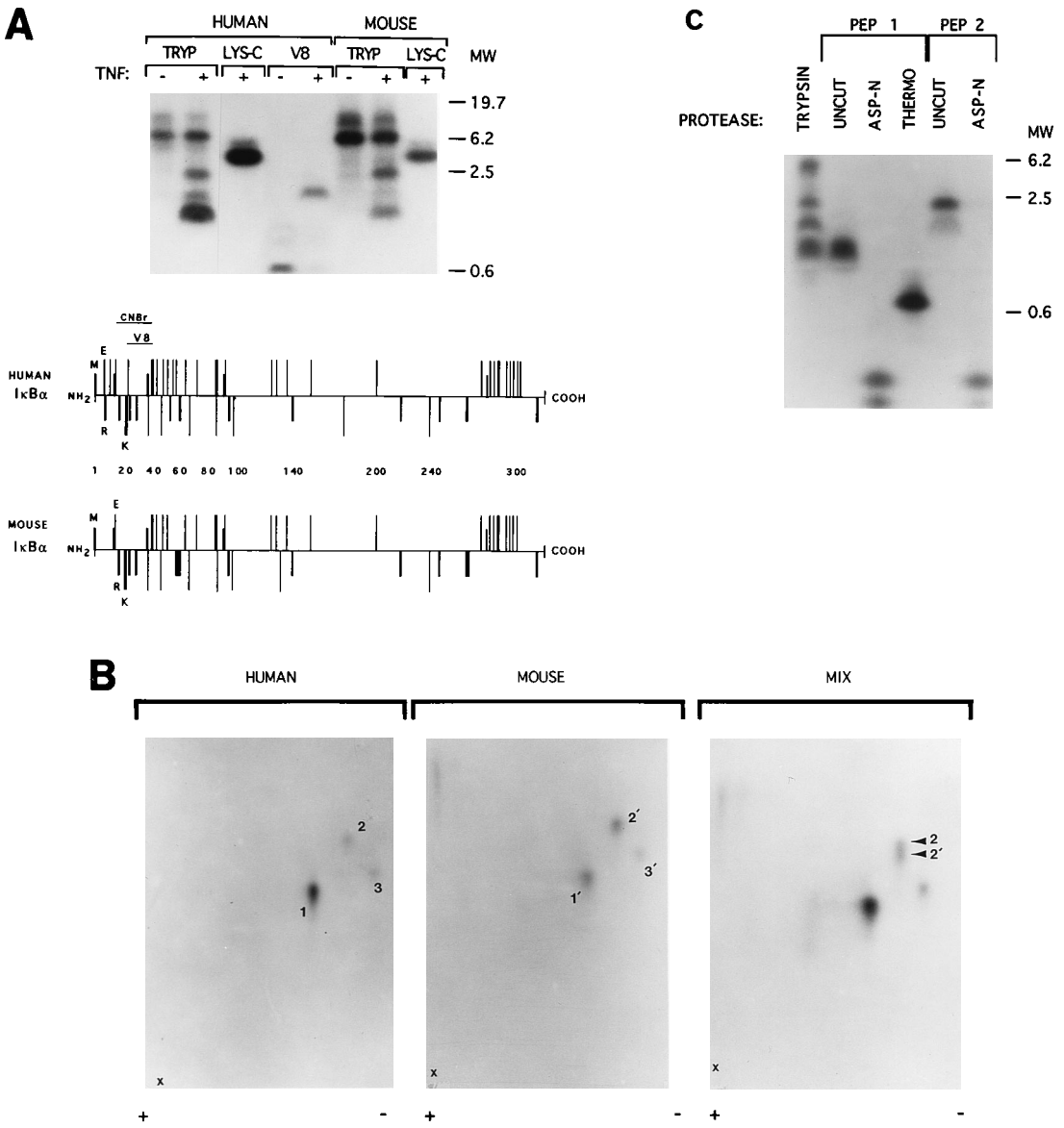

FIG. 1. Mapping the inducible phosphorylation sites of IκBα. (A) In the top panel, HeLa and NIH 3T3 cells labeled with ^{32}P for 4 h were incubated with 100 μM Ac-LLnL-CHO for 90 min prior to stimulation with TNF-α (10 ng/ml). After 10 min, the cells were lysed, and IκBα was immunoprecipitated, separated by SDS-PAGE, and transferred to nitrocellulose membranes. The phosphorylated IκBα bands were identified by autoradiography, excised, and digested with either trypsin (TRYP; 50 μg), LYS-C (25 μg), or V8 protease (50 μg), as indicated. The resulting phosphopeptides were separated on a Tris-Tricine gel and visualized by autoradiography for 3 to 4 days at −80°C with an intensifying screen. Molecular masses (MW) are indicated in kilodaltons. The bottom panel shows a schematic depicting the location of the residues recognized by the different proteases and CNBr in human and mouse IκBαs. The locations of the CNBr- and V8-generated fragments containing the inducible phosphorylation sites are indicated. Note that approximately four to five times more counts per minute were loaded in the TNFα + lanes than the − lanes except for the V8 digest lanes, in which cases equal numbers of counts per minute were loaded. (B) Phosphopeptide maps of human and mouse IκBα isolated from TNFα-stimulated cells as described above. ^{32}P-labeled IκBαs were digested with trypsin, and the tryptic digests were subjected to two-dimensional separation (horizontal, high-voltage electrophoresis, vertical, thin-layer chromatography). A total of 1,500 cpm (human IκBα), 1,100 cpm (mouse IκBα), and 750 cpm each of human and mouse IκBαs (mix) was applied. (C) Tryptic phosphopeptides (PEP) 1 and 2 of human IκBα were isolated from the thin-layer plates and digested with ASP-N or thermolysin, as indicated. Samples of the undigested and the digested peptides were separated on a Tris-Tricine gel. A tryptic digest of human IκBα from TNF-α-stimulated cells was used as a reference. Molecular masses (MW) are indicated in kilodaltons.

tates, or thermolysin, which cleaves amino terminal to hydrophobic residues. The different phosphopeptides were fractionated on high-resolution Tris-Tricine gels. Phosphopeptides 1 and 2 comigrated with the 1.0- and 2.4-kDa tryptic phosphopeptides, respectively (Fig. 1C), whereas phosphopeptide 3 comigrated with the 1.8-kDa tryptic phosphopeptide (data not shown). Digestion of either phosphopeptide 1 or 2 with ASP-N generated phosphopeptides migrating around 0.4 kDa, whereas thermolysin cleavage of phosphopeptide 1 generated a 0.7-kDa phosphopeptide (Fig. 1C).

Collectively, these results indicate that tryptic phosphopeptide 2 is most likely due to cleavage at R-24 and K-47, while the overlapping tryptic phosphopeptide 1 is most likely due to cleavage at R-29 and K-38. These assignments are consistent with the sizes of both phosphopeptides and the differences in hydrophobicity between the human and mouse versions of

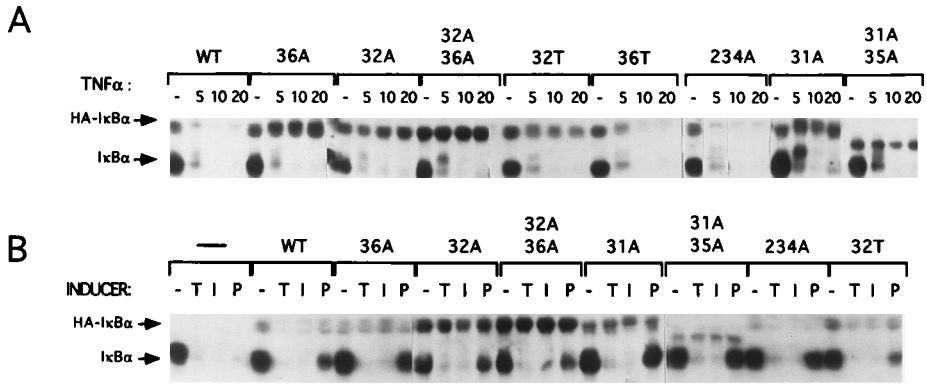

FIG. 2. Time course analysis of WT and mutant IκBα degradation. (A) Cell pools stably expressing the indicated HA-IκBα constructs were stimulated with TNF-α (10 ng/ml). At the indicated times (minutes), whole cell lysates were prepared, and 30-μg samples were separated by SDS-PAGE and transferred to Immobilon-P membranes. The abundances of endogenous and exogenous (HA-tagged) IκBαs were determined by immunoblotting with a polyclonal IκBα antiserum and detection with enhanced chemical luminescence. Note that HA-IκBα(31A/35A) migrates faster than the other HA-IκBαs. (B) The different cell pools expressing WT and mutant HA-IκBαs and untransfected HeLa cells were incubated for 25 min with TNF-α (T; 10 ng/ml) or IL-1α (I; 2 ng/ml) or for 30 min with TPA (P; 100 ng/ml). Whole cell lysates were prepared, fractionated by SDS-PAGE, and immunoblotted with anti-IκBα antiserum as described above.

phosphopeptide 2. Within the region covered by these peptides, the two IκBαs differ only at position 26, where the human version contains a leucine and the mouse polypeptide contains a valine. Phosphopeptide 3, on the other hand, is of identical hydrophobicity in both species and therefore is most likely due to cleavage at R-29 and K-47. The sizes of the thermolysin- and ASP-N-generated phosphopeptides are also consistent with these assignments. Thermolysin most likely cleaves at M-37, generating a peptide of seven amino acids. The ASP-N-generated peptides are most likely due to cleavage at D-31 and D-35. Therefore, the only sites on IκBα, whose phosphorylation is induced by TNF-α, that are compatible with all of these results are S-32 and S-36.

Mutant analysis of IκBα degradation. To confirm the assignment of S-32 and S-36 as the inducible phosphoacceptor sites and examine their roles, they were replaced by either alanines (32A, 36A, and 32A/36A) or threonines (32T and 36T). In addition, the aspartates which precede both serines were altered either singularly (31A and 35G) or doubly (31A/35A). The WT and mutant IκBα alleles were tagged with the influenza virus HA epitope (51) and stably transfected into HeLa S3 cells. Pools of G418-resistant cells were established, and after incubation with TNF-α, the levels of endogenous and exogenous (HA-tagged) IκBα were examined by immunoblotting (Fig. 2A). In all cases, endogenous IκBα was degraded rapidly and very little protein was found 5 min after TNF-α addition. WT HA-IκBα was degraded with kinetics similar to that for endogenous IκBα. Mutant 234A, generated as a control, was also rapidly degraded. On the other hand, mutants 32A, 36A, and 32A/36A were not degraded upon cell stimulation with TNF-α. Surprisingly, mutants 32T and 36T were also defective in their responses to TNF-α. While mutant 36T was degraded more slowly than WT HA-IκBα, 32T was degraded with extremely slow kinetics. Interestingly, 31A and 31A/35A were also stable after cell stimulation with TNF-α (Fig. 2A). By contrast, mutant 35G was degraded with nearly WT kinetics (data not shown). Other mutants, in which S-262 and T-263, in the putative protein kinase C site (25), were replaced by alanines, were degraded with WT kinetics. To determine whether the different mutations affected IκBα degradation in response to other inducers, we compared the degradation kinetics of the mutants after cell stimulation with either TNF-α, IL-1, or TPA (Fig. 2B). The potent inducers, TNF-α and IL-1, caused almost complete degradation of endogenous and HA-tagged WT and 234A IκBαs. All of the other mutants that were resistant to TNF-α-induced degradation were also refractory to IL-1-induced degradation. Similar results were obtained with the weaker inducer, TPA. Interestingly, TPA induced nearly complete degradation of endogenous IκBα in the parental cells but only partial degradation in all of the HA-tagged IκBα-expressing cell pools, including those that express WT and 234A HA-IκBαs. Perhaps a weaker inducer such as TPA does not fully activate all of the available IκBα kinase and the presence of excess substrate renders the kinase or another component rate limiting for IκBα degradation.

To demonstrate that the WT and mutant IκBαs can associate with NF-κB and block its binding to DNA, nuclear extracts of TNF-α-stimulated HeLa cells were incubated with either nonprogrammed wheat germ lysate or lysates in which WT HA-IκBα and the different mutants were translated. The level of NF-κB DNA binding activity, which in these extracts is composed mostly of p50-p65 heterodimers (15), was determined by electrophoretic mobility shift assay (Fig. 3A). The different mutants were fully capable of inhibiting NF-κB binding to DNA. To show that the mutant IκBαs associate with p65 (RelA) in vivo, we immunoprecipitated WT and mutant HA-IκBαs from cell lysates with anti-HA under conditions that preserve the integrity of IκB–NF-κB complexes (16). After the immune complexes were disrupted and separated on a denaturing gel, they were transferred to membranes that were probed with either anti-IκBα or anti-p65 antibody. As shown in Fig. 3B, both the WT and 32A/36A, 32A, 35A, and 32T mutant HA-IκBαs were associated with p65 (RelA) in nonstimulated cells.

Substitutions at serines 32 and 36 block inducible IκBα phosphorylation. To determine the effects of the mutations on IκBα phosphorylation, cell pools expressing the mutants were metabolically labeled with $^{32}P_i$, and endogenous and HA-tagged IκBα proteins were isolated by immunoprecipitation before and after cell stimulation with TNF-α. None of the single amino acid substitutions (31A, 32A, 32T, 35G, 36A, and 36T) prevented inducible IκBα phosphorylation (data not shown). Only the double mutants 32A/36A (Fig. 4A) and 31A/35A (data not shown) were no longer inducibly phosphorylated in response to TNF-α. Most importantly, two-dimensional mapping revealed that the three TNF-α-inducible tryptic phosphopeptides were no longer detected in digests of HA-IκBα(32A/36A) (Fig. 4A) or HA-IκBα(31A/35A) (data not

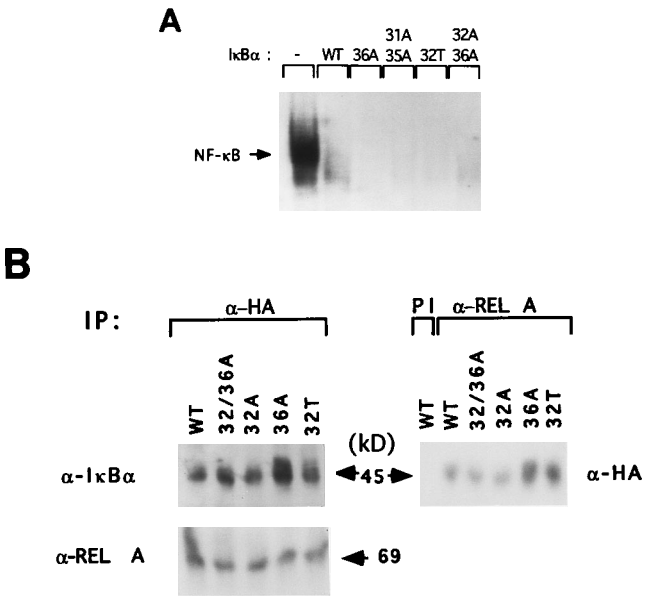

FIG. 3. Mutant IκBαs can still bind to NF-κB. (A) Equal amounts of WT and mutant IκBαs synthesized in a wheat germ-coupled transcription-translation system or an equivalent volume of unprogrammed wheat germ lysate (−) were added to a nuclear extract (5 μg of protein) prepared from TNF-α-stimulated HeLa cells. The mixtures were incubated with a κB probe, and DNA binding was determined by electrophoretic mobility shift assay. (B) Cytoplasmic extracts (400 μg) from WT and the indicated mutant IκBα cell pools were immunoprecipitated (IP) either with monoclonal antibody 12CA5 (α-HA) or with anti-RelA antibody 519 or its preimmune serum (PI), as indicated. Immunopellets were fractionated by SDS-PAGE and transferred to Immobilon-P membranes for immunoblot analysis with anti-IκBα, anti-RelA (anti-p65-C), and anti-HA antibodies, as indicated.

shown). The phosphopeptides that were still present in digests of these double mutants corresponded to the constitutive phosphorylation sites, whose level of phosphorylation is considerably lower than that of the inducible sites. [The slight differences in the intensities of these phosphopeptides in the HA-IκBα(32A/36A) samples are due to loading of different amounts of radioactivity.] These results indicate that S-32 and S-36 are the only inducible phosphorylation sites.

As indicated above, the single mutants 32T and 36T are still inducibly phosphorylated. To determine whether these substitutions result in appearance of phosphothreonine in IκBα and thus obtain further evidence for the use of these phosphoacceptor sites, HA-IκBα(32T) and HA-IκBα(36T) from TNF-α-stimulated cells were subjected to phosphoamino acid analysis. After two-dimensional separation of tryptic digests of these and WT HA-IκBα, the inducible phosphopeptides were recovered and subjected to acid hydrolysis. Figure 4B indicates that all of the phosphopeptides thus analyzed, either from WT IκBα (peptides 1, 2, and 3) or mutant IκBαs (only peptide 1), contained only phosphoserine; no phosphothreonine could be detected. Thus, the IκBα kinase appears to have a very strong preference for serines.

Similar phosphoacceptors are required for inducible IκBβ degradation. Comparison of IκBα (25) and IκBβ (44) in the regions outside their ankyrin repeats revealed a striking similarity in the sequence surrounding S-32 and S-36 of IκBα (Fig. 5A). Also of note are the conserved lysines and aspartates at positions 22 and 31 of IκBα, respectively. These similarities suggest that S-19 and S-23 of IκBβ may also be phosphoacceptor sites for the putative IκB kinase. To examine this possibility, expression vectors encoding WT HA-IκBβ and single (19A and 23A) and double (19A/23A) substitution mutants were constructed and stably transfected into HeLa cells. G418-resistant cell pools were generated and used for further analysis. Immunoblotting with IκBβ-specific antibodies (44) indicated that cell stimulation with either TNF-α or IL-1 resulted in degradation of endogenous IκBβ or WT HA-IκBβ within 30 min (Fig. 5B). All three substitution mutants were resistant to inducible degradation. Of note is the stabilizing effect by pretreatment of the cells with the peptide aldehyde proteasome inhibitor Z-LLF-CHO (49) (Fig. 5B, lane I+F), suggesting involvement of the proteasome in inducible IκBβ degradation, as already shown for IκBα. As shown in Fig. 5C, the different IκBβ mutants were all functional in inhibition of NF-κB DNA binding activity.

Phosphorylation-dependent ubiquitination of IκBα. As proteasome-mediated degradation depends on polyubiquitination of the target protein (reviewed in references 21, 22, and 28), it is expected that phosphorylation of IκBα leads to its ubiquitination. Recently, using an in vitro system, Chen et al. (12) demonstrated that phosphorylation of IκBα at S-32 and S-36 is required for its ubiquitination. However, no evidence was provided to indicate that signal-induced phosphorylation of IκBα causes its ubiquitination in living cells. To examine this important point, we stimulated cell pools expressing either WT or 32A/36A HA-IκBα with TNF-α in the absence or presence of the proteasome inhibitor Z-LLF-CHO. The tagged IκBα proteins were immunoprecipitated with anti-HA, and the immune complexes were separated on a denaturing gel and transferred to Immobilon-P membranes. Parallel blots were probed with either anti-HA or an antibody raised against a C-terminal IκBα peptide. TNF-α stimulation of cells that were preincubated with the proteasome inhibitor resulted in appearance of high-molecular-weight forms of WT HA-IκBα but not of HA-IκBα(32A/36A) (Fig. 6A). To examine whether these high-molecular-weight forms, migrating above a size of 200 kDa, are due to polyubiquitination, WT and HA-IκBα(32A/36A) were precipitated with anti-HA from Z-LLF-CHO-treated cells that had been incubated in the absence or presence of TNF-α. Immunoblot analysis of these immune complexes with the C-terminal IκBα antibody revealed the presence of high-molecular-weight forms of WT HA-IκBα but not HA-IκBα(32A/36A) in TNF-α-treated cells (Fig. 6B). Stripping the blot and reprobing with affinity-purified antiubiquitin antibody (24) revealed that the high-molecular-weight HA-IκBα forms contain ubiquitin chains. Thus, cell stimulation with TNF-α results in polyubiquitination of IκBα, a modification that is dependent on its inducible phosphorylation at S-32 and S-36.

Previously it was shown that phosphorylation of IκBα does not induce its dissociation from NF-κB complexes (2, 16, 17, 32, 38, 46). We examined whether ubiquitination of IκBα results in dissociation from NF-κB complexes. These complexes were isolated by immunoprecipitation with anti-p65 antibodies from nonstimulated and TNF-α-stimulated cells that were preincubated with Z-LLF-CHO. Following separation on a denaturing gel and transfer to a membrane, immunoblotting of the p65-containing complexes with the C-terminal anti-IκBα antibody revealed that the high-molecular-weight forms of IκBα, whose appearance was induced by TNF-α, were still associated with p65-containing complexes (Fig. 6C). Thus, ubiquitination of IκBα also does not result in its dissociation from NF-κB.

Substitution for lysines 21 and 22 interferes with IκBα ubiquitination and degradation. Experiments similar to those described above but using anti-IκBβ antibodies indicated that IκBβ is also subjected to inducible ubiquitination (data not shown). As protein ubiquitination occurs on lysine residues (28), we examined IκBα and IκBβ for occurrence of conserved

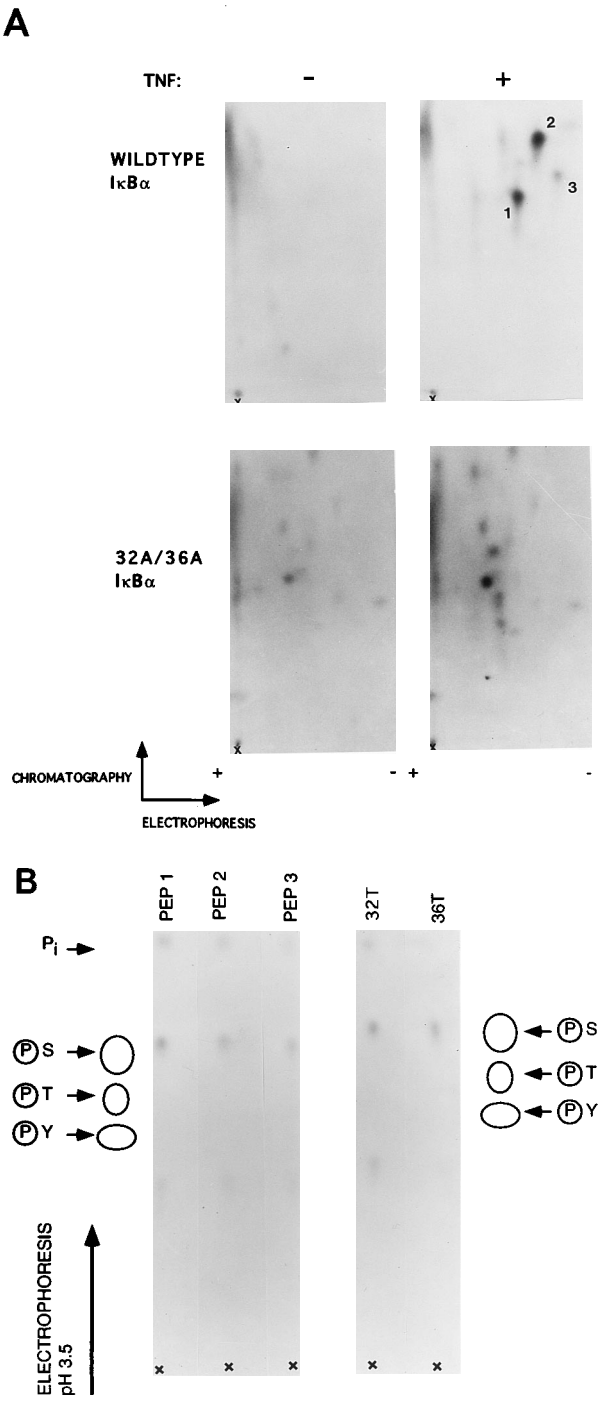

FIG. 4. The inducible phosphorylation sites of IκBα are serines 32 and 36. (A) Phosphopeptide maps of WT HA-IκBα and HA-IκBα(32A/36A). Pools of HeLa cells expressing either form of HA-IκBα were metabolically labeled with ^{32}P, treated with Ac-LLnL-CHO, and left unstimulated or stimulated with TNF-α (10 ng/ml) for 10 min, and then the cells were lysed and the ^{32}P-labeled IκBαs were isolated as described for Fig. 1. The excised bands corresponding to the HA-IκBαs were digested with trypsin and analyzed by two-dimensional phosphopeptide mapping. Each plate was loaded with 1,200 cpm of ^{32}P-IκBα except for HA-IκBα(32A/36A) from nonstimulated cells, of which only 850 cpm was loaded. (B) Phosphoamino acid analysis of phosphopeptides (PEP) that contain TNF-α-inducible phosphorylation sites of WT, 32T, and 36T IκBαs. The different ^{32}P-labeled IκBα proteins were isolated from TNF-α-stimulated cells and subjected to tryptic phosphopeptide mapping. The phosphopeptides were isolated from tryptic phosphopeptide maps and subjected to phosphoamino acid analysis. In the case of WT IκBα, we used phosphopeptides 1, 2, and 3 (see panel A), and in the case of the mutants, we isolated the major inducible phosphopeptide (peptide 1). The hydrolysates were separated by high-voltage electrophoresis at pH 3.5. The positions of nonradioactive phosphoamino acid markers loaded on the same plates are indicated. The position of free phosphate is also marked.

lysine residues around their phosphoacceptor sites. The only conserved lysine is K-22 in IκBα and K-9 in IκBβ (Fig. 5A). To test whether this residue is the likely site of IκBα ubiquitination and, if so, to determine the functional importance of this modification, we replaced K-22 with an arginine. However, as residue 21 is also a lysine and frequently in the absence of the primary recognition site, the ubiquitination machinery acts on adjacent secondary sites (see reference 47 for an example), we also replaced K-21 with an arginine. Pools of HeLa cells stably expressing the 21R/22R mutant were used to analyze the effects of these substitutions on IκBα ubiquitination, degradation, and phosphorylation. Immunoprecipitation of HA-IκBα(21R/22R) from unstimulated and TNF-α-stimulated cells followed by immunoblot analysis with the C-terminal anti-IκBα antibody revealed that the extent of its conversion to higher-molecular-weight forms was considerably reduced (4.5-fold) in comparison with WT HA-IκBα (Fig. 7A). Stripping and reprobing the blot with the antiubiquitin antibody confirmed the presence of ubiquitin in these more slowly migrating forms and revealed a similar decrease (threefold) in the efficiency of HA-IκBα(21R/22R) ubiquitination in comparison with its WT counterpart (data not shown).

To determine whether the reduced efficiency of ubiquitination affected the kinetics of HA-IκBα(21R/22R) degradation, we measured the abundance of mutant and WT IκBα after cell stimulation with TNF-α. As shown in Fig. 7B and C, TNF-α induced the degradation of HA-IκBα(21R/22R), but with much slower kinetics in comparison with both endogenous IκBα in the same cell pool and WT HA-IκBα. While the half-lives of endogenous IκBα and WT HA-IκBα were 2 to 2.5 min, the half-life of HA-IκBα(21R/22R) was 8.5 min. Despite the marked effects on inducible ubiquitination and degradation of IκBα, the dual 21R/22R substitution did not affect its phosphorylation. Phosphopeptide mapping of either HA-IκBα (21R/22R) or endogenous IκBα digested with V8 protease revealed the presence of the same 2-kDa phosphopeptide containing the TNF-α-inducible phosphorylation sites (Fig. 7D).

DISCUSSION

A common mechanism, phosphorylation-induced ubiquitination, for IκB degradation. The use of protease inhibitors provided evidence that activation of the transcription factor NF-κB in response to different extracellular stimuli depends on degradation of IκBα (1, 16, 17, 32, 38, 39, 46). It was also shown that IκBα degradation is preceded by its inducible phosphorylation (1, 17, 32, 38, 46), and mutant analysis suggested that phosphorylation of IκBα targets it for degradation (10, 45, 50). Although we identified two serine residues, S-32 and S-36, whose replacement by nonphosphorylatable alanines prevents inducible IκBα degradation, no direct evidence that these are indeed the sites of inducible IκBα phosphorylation was provided (9, 10, 45, 50). For instance, these substitutions could either affect IκBα phosphorylation at other sites or prevent its degradation by altering its structure. Using phosphopeptide mapping and phosphoamino acid analysis, we have unequivocally identified S-32 and S-36 as the only sites of inducible IκBα phosphorylation. Replacement of either serine with an alanine is sufficient to block IκBα degradation. As inducible IκBα phosphorylation is prevented only by the dual 32A/36A substitution but not by single 32A or 36A substitution (data not

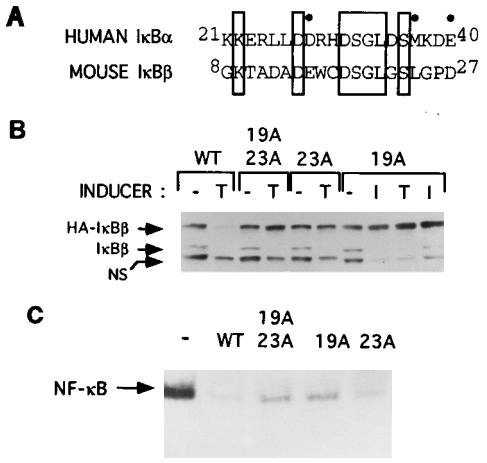

FIG. 5. Inhibition of IκBβ degradation by substitution of serines 19 and 23. (A) Comparison of the IκBα and IκBβ sequences in the region surrounding the inducible phosphorylation sites of IκBα. Boxes, identical residues; dots, similar residues. (B) Pools of HeLa cells stably transfected with expression vectors encoding HA-tagged WT and mutant IκBβs were incubated in the absence or presence of TNF-α (T; 10 ng/ml) or IL-1α (I; 2 ng/ml) for 30 min. In one case, the last lane, the cells were preincubated with the proteasome inhibitor Z-LLF-CHO (10 μM) prior to addition of IL-1α. Cell lysates were separated by SDS-PAGE, transferred to Immobilon-P membranes, and analyzed for IκBβ abundance by immunoblotting with anti-IκBβ (44). The migration positions of endogenous and exogenous (HA-tagged) IκBβ and a cross-reacting protein (NS [nonspecific]) are indicated. (C) Mutant IκBβs inhibit NF-κB DNA binding. Equal amounts of WT and the indicated mutant IκBβs synthesized in a wheat germ-coupled transcription-translation system or the equivalent amount volume of unprogrammed wheat germ lysate (−) were added to a nuclear extract (5 μg of protein) prepared as described for Fig. 3A. The mixtures were incubated with a κB probe, and DNA binding was determined by electrophoretic mobility shift assay.

shown), it seems that phosphorylation of a single site is sufficient for targeting IκBα to the degradation pathway or that introduction of a hydrophobic residue into one of these positions may have an additional adverse effect on IκBα degradation (see below). The same serines (S-19 and S-23) are conserved in IκBβ, and substitution with alanines also prevents inducible IκBβ degradation. These results strongly suggest that IκBα and IκBβ are regulated through the same mechanism.

IκB phosphorylation converts these proteins into efficient substrates for the protein ubiquitination machinery. Upon cell stimulation, IκBα and IκBβ are rapidly modified to yield high-molecular-weight forms containing ubiquitin chains. As recently shown (12), detection of ubiquitinated IκB requires preincubation with a proteasome inhibitor, consistent with the notion that polyubiquitinated proteins are very efficient substrates for proteasomal degradation (22, 28). As the bulk of ubiquitinated IκBα and IκBβ migrates more slowly than 200 kDa, their ubiquitination is rather extensive and many of the modified molecules contain 20 or more conjugated ubiquitin molecules. As the phosphorylation-defective mutant IκBα (32A/36A) fails to undergo inducible ubiquitination, it can be concluded that phosphorylation of IκBα is required for its ubiquitination. Chen et al. (12) and Alkalay et al. (3), using cell-free ubiquitin-conjugating systems, have recently arrived at similar conclusions. Most likely, the phosphorylated IκB is recognized by a specific ubiquitin protein ligase (E3 [13]).

As the phosphorylated forms of both IκBα and IκBβ are subject to inducible ubiquitination, it is likely that the ubiquitination machinery recognizes a common feature of both proteins. Because ubiquitin ligation occurs on lysine residues, we searched the two proteins for conserved lysines in their N-terminal domains, the sites of inducible phosphorylation. Only one such lysine, K-22 in IκBα and K-9 in IκBβ, was identified. Replacement of K-22 and the adjacent K-21 with arginines results in a considerable decrease in the efficiency of IκBα ubiquitination and slows down its degradation rate. These substitutions, however, have no adverse effects on inducible IκBα phosphorylation. It is not surprising, however, that IκBα(21R/22R) is still ubiquitinated following cell stimulation. Quite

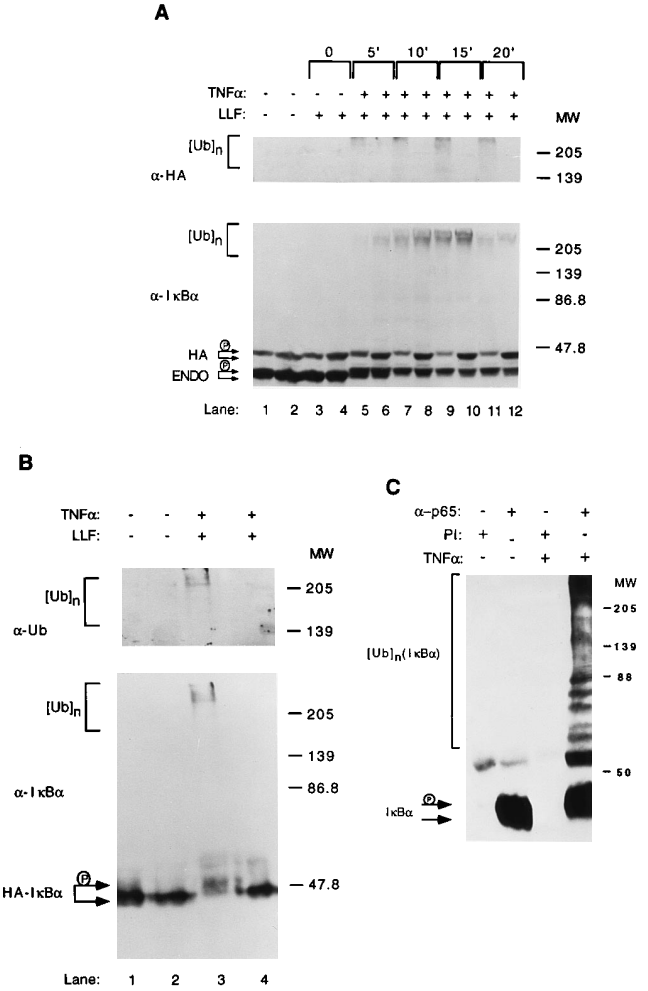

FIG. 6. Inducible IκBα phosphorylation leads to its polyubiquitination. (A) Cell pools expressing WT HA-IκBα (odd-numbered lanes) or HA-IκBα(32A/36A) (even-numbered lanes) were either left untreated or preincubated with Z-LLF-CHO (10 μM) for 60 min and stimulated with TNF-α (10 ng/ml) as indicated. At the indicated time points, whole cell lysates were prepared, fractionated by SDS-PAGE, and transferred to Immobilon-P membranes. The blots were probed with either an anti-IκBα C-terminal affinity-purified antibody (bottom panel) or anti-HA antibody (upper panel). (B) Cells were treated as described above, and lysates were prepared 12 min after TNF-α stimulation and immunoprecipitated with the anti-HA antibody. The immune complexes were disrupted, fractionated by SDS-PAGE, and transferred to an Immobilon-P membrane. The blot was first probed with the anti-IκBα C-terminal antibody (bottom panel). It was then stripped and reprobed with affinity-purified antiubiquitin antibody (upper panel). The migration positions of endogenous IκBα (ENDO), HA-tagged IκBαs (HA), and polyubiquitinated IκBαs [$(Ub)_n$] are indicated. (C) HeLa cells preincubated with Z-LLF-CHO for 60 min were left unstimulated (−) or stimulated with TNF-α (+). After 12 min, whole cell lysates were prepared and subjected to immunoprecipitation with either anti-p65 (antibody 519) or preimmune serum (PI). The immune complexes were disrupted, fractionated by SDS-PAGE, and transferred to an Immobilon-P membrane. The membrane was probed with the anti-IκBα C-terminal antibody. The different forms of IκBα are marked as in panel B. Molecular masses (MW) are indicated in kilodaltons.

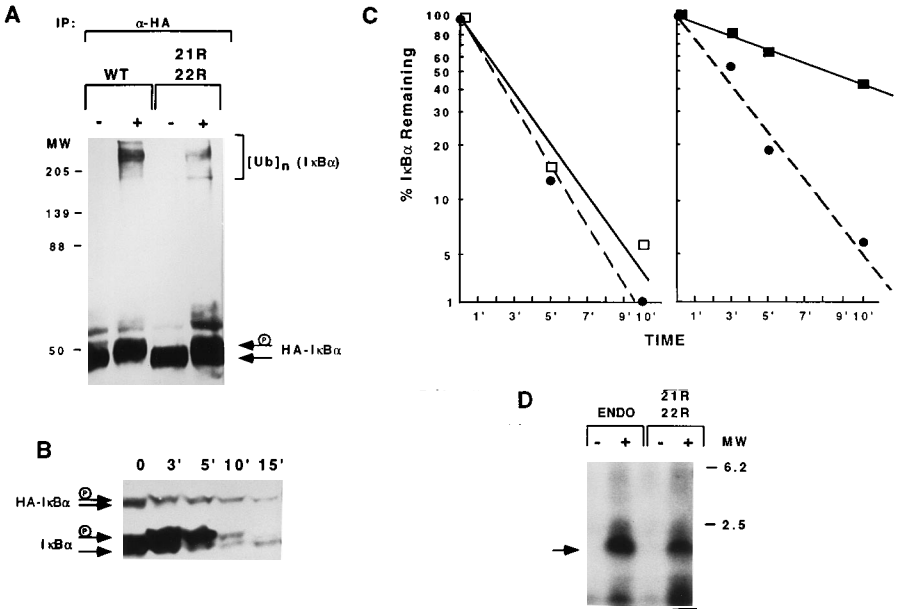

FIG. 7. Substitution of lysines 21 and 22 affects the ubiquitination and degradation of IκBα but not its phosphorylation. (A) Cell pools expressing WT HA-IκBα or HA-IκBα(21R/22R) were preincubated with Z-LLF-CHO and left unstimulated (−) or stimulated with TNFα (+). After 12 min, cell lysates were prepared and the HA-IκBαs were immunoprecipitated (IP) with the anti-HA antibody (α-HA). Immune complexes were disrupted, fractionated by SDS-PAGE, and transferred to an Immobilon-P membrane for immunoblot analysis with an anti-IκBα C-terminal antibody. The migration positions of the HA-tagged IκBαs and polyubiquitinated HA-IκBαs [$(Ub)_n$] are indicated. (B) Cell pools expressing HA-IκBα(21R/22R) were stimulated with TNF-α, and at the indicated times (minutes), lysates were prepared and subjected to immunoblot analysis as described for Fig. 2A except that anti-IκBα C-terminal antibody c-21 was used to probe the blot. (C) The relative amounts of WT HA-IκBα (□), HA-IκBα(21R/22R) (■), and endogenous IκBα (●) were determined by densitometric scanning of the fluorograms shown above and in Fig. 2A and plotted against the length of exposure to TNF-α. (D) Pools of cells expressing HA-IBα(21R/22R) were metabolically labeled with $^{32}P_i$ for 4 h, pretreated with Ac-LLnL-CHO, and left unstimulated (−) or stimulated with TNF-α (+) for 10 min, and then the cells were lysed and the ^{32}P-labeled IκBαs were isolated as described for Fig. 1. The excised bands corresponding to HA-IκBα(21R/22R) and endogenous IκBα (ENDO) were digested with V8 protease and analyzed on a high-resolution TTE gel. The position of the inducible 2-kDa phosphopeptide is indicated. In nonstimulated lanes, 200 cpm was loaded; 650 cpm was loaded on the TNF-α-stimulated lanes. Molecular masses (MW) are indicated in kilodaltons.

frequently, inactivation of one ubiquitination site results in the utilization of alternative sites by the ubiquitination machinery (47). At this point, we do not know whether K-21 is a physiological ubiquitination site, but since only K-22 is conserved in IκBβ, it is probably the primary ubiquitination site. Nevertheless, it is clear that ubiquitination of IκB is necessary for tagging it for degradation by the proteasome. These conclusions are consistent with many other observations on degradation of polyubiquitinated proteins (13) and the inhibition of IκB degradation by proteasome inhibitors.

Single or multiple IκB kinases? In response to stimulation of cells with TNF-α, IL-1, or TPA, IκBα is rapidly phosphorylated on two sites, S-32 and S-36. Substitution of either serine or their homologs in IκBβ (S-19 and S-23) with nonphosphorylatable alanines is sufficient to prevent inducible IκB degradation. These results suggest that IκBα and IκBβ may be substrates for the same IκB kinase or a small family of IκB kinases. In 70Z/3 pre-B cells, IL-1 and LPS are effective inducers of both IκBα and IκBβ degradation, whereas TPA induces IκBα but not IκBβ degradation (44). Similar differences were found in HeLa cells, in which TNF-α and IL-1 induced both IκBα and IκBβ degradation but TPA induced only IκBα degradation (data not shown). While these results were previously interpreted to suggest fundamental differences in the regulation of IκBα and IκBβ (43, 44), there is also another, more parsimonious explanation. Inducible IκBβ degradation occurs with considerably slower kinetics than IκBα degradation (15a, 44), probably because it is phosphorylated less efficiently by the IκB kinase. The mutational analysis described above and elsewhere suggests that the rate-limiting step in IκBα degradation is its phosphorylation. Once phosphorylated, IκBα is rapidly ubiquitinated and degraded. Thus, the efficiency of IκB phosphorylation dictates how fast it will be degraded. A weaker kinase activator may still induce substantial yet slow IκBα degradation while hardly having an effect on phosphorylation and turnover of the less favored substrate, IκBβ. Thus, we suggest that TPA, which is not as efficient as IL-1, TNF-α, or LPS in induction of NF-κB binding activity, is simply a weaker activator of the IκB kinase. In agreement with this interpretation, we find that even a small increase in the level of IκBα in cells expressing HA-IκBα is sufficient to decrease the extent of IκBα degradation in response to TPA but not following stimulation with IL-1 or TNF-α (Fig. 2B).

The differences in the efficiency of IκBα and IκBβ phosphorylation may be dictated by residues that surround their phosphoacceptor sites. Interestingly, S-32 and S-36 of IκBα are both preceded by aspartates, one of which, D-31, is conserved in IκBβ. Substitution of D-31 by an alanine is sufficient to block IκBα degradation. However, as with the single substitution of the phosphoacceptor sites (i.e., 32A and 36A), the 31A mutant is still subject to inducible phosphorylation (15a). Nevertheless, the major effect of the 31A substitution is likely to be on IκBα phosphorylation because the double mutant 31A/35A, just like the double mutant 32A/36A, is no longer subjected to inducible phosphorylation (15a). Unlike D-31, D-35 is not conserved in IκBβ, in which the equivalent position is G-22. To examine the significance of this change, we expressed a 35G mutant of IκBα. This mutant, however, was degraded with WT kinetics (15a). Thus, it is unlikely that the D-to-G substitution accounts for the slower kinetics of IκBβ degradation. Probably

some of the other residues that differ between the N-terminal regions of IκBα and IκBβ are responsible for the differences in their degradation kinetics.

Degradation of IκBα was also affected by replacement of S-32 and S-36 by threonines. While mutant 32T was refractory to inducible degradation, mutant 36T was degraded more slowly than the WT protein. When isolated from TNF-α-stimulated cells and subjected to phosphoamino acid analysis, neither 32T nor 36T was found to contain detectable amounts of phosphothreonine. These results indicate that the IκB kinase has very strong preference for serine as a phosphoacceptor. In addition, these results suggest that S-32 is the major regulatory phosphorylation site and that the more severe effect of the 36A mutation in comparison with the 36T mutation on IκBα degradation could be due to a secondary effect of having a hydrophobic residue at position 36 rather than the mere absence of phosphorylation. The strong preference of serine as a phosphoacceptor by the IκB kinase is unusual, as most known serine kinases can also use threonine, albeit somewhat less efficiently. The only known protein kinase with a strong preference for serine is protein kinase A (29). However, since protein kinase A is activated by cyclic AMP but not by TPA, TNF-α, or IL-1, it is unlikely to be the IκB kinase. Anyhow, the strong preference for serine is likely to be useful in identification of the physiological IκB kinase. Another feature that may help in identification of the IκB kinase is the apparent preference for negatively charged residues N terminal to the phosphoacceptor site. Although their specific roles have not been tested, both IκBα and IκBβ have two additional conserved acidic residues that precede S-32 in IκBα and S-19 in IκBβ. This feature resembles the sequence of casein kinase I phosphorylation sites (18). However, casein kinase I does not display a strong preference for serine over threonine, and its activity is constitutive (48).

Several protein kinases were previously proposed as IκB kinases. It was shown that phosphorylation of IκBα in vitro by either protein kinase A or protein kinase C prevents its binding to NF-κB (19). Dissociation of IκBα–NF-κB complexes was also reported to be induced by treatment of cell extracts with double-stranded RNA-dependent protein kinase (30). In vivo, phosphorylation of IκBα does not result in its dissociation from NF-κB (2, 16, 17, 32, 38, 46), nor do the inducible phosphorylation sites resemble known protein kinase A or C sites. Raf-1 was also suggested as an IκB kinase (31). However, Raf-1 in many cell types, including HeLa cells, is activated by growth factors but not by TNF-α (37). Furthermore, the only known Raf-1 substrates are MEK1 and MEK2, and their activation sites recognized by Raf-1 do not resemble the inducible IκB phosphorylation sites (1). In summary, despite the long list of putative IκB kinases, the physiologically relevant kinase(s) responsible for inducible phosphorylation of IκBα and IκBβ remains to be identified. It is clear, however, that induction of IκB phosphorylation represents the first and perhaps most critical step toward NF-κB activation.

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