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Phosphorylation of IκBα in the C-Terminal PEST Domain by Casein Kinase II Affects Intrinsic Protein Stability

RONGTUAN LIN,[1] PIERRE BEAUPARLANT,[1] CONSTANTIN MAKRIS,[2] SYLVAIN MELOCHE,[2] AND JOHN HISCOTT[1]*

Terry Fox Molecular Oncology Group, Lady Davis Institute for Medical Research, Sir Mortimer B. Davis Jewish General Hospital, and Departments of Microbiology and Immunology and Medicine, McGill University, Montreal, Quebec H3T 1E2,[1] and Centre de Recherche, Hôtel Dieu de Montréal, and Department of Pharmacology, University of Montreal, Montreal, Quebec H2W 1T8,[2] Canada

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The NF-κB/Rel transcription factors participate in the activation of immune system regulatory genes and viral early genes including the human immunodeficiency virus type 1 long terminal repeat. NF-κB/Rel proteins are coupled to inhibitory molecules, collectively termed IκB, which are responsible for cytoplasmic retention of NF-κB. Cell activation leads to the phosphorylation and degradation of IκBα, permitting NF-κB/Rel translocation to the nucleus and target gene activation. To further characterize the signaling events that contribute to IκBα phosphorylation, a kinase activity was isolated from Jurkat T cells that specifically interacted with IκBα in an affinity chromatography step and phosphorylated IκBα with high specificity in vitro. By using an in-gel kinase assay with recombinant IκBα as substrate, two forms of the kinase (43 and 38 kDa) were identified. Biochemical criteria and immunological cross-reactivity identified the kinase activity as the alpha catalytic subunit of casein kinase II (CKII). Deletion mutants of IκBα (Δ1 to Δ4) localized phosphorylation to the C-terminal PEST domain of IκBα. Point mutation of residues T-291, S-283, and T-299 dramatically reduced phosphorylation of IκBα by the kinase in vitro. NIH-3T3 cells that stably expressed wild-type IκBα (wtIκB), double-point-mutated IκBα(T291A, S283A), or triple-point-mutated IκBα(T291A, S283A, T299A) under the control of the tetracycline-responsive promoter were generated. Constitutive phosphorylation of the triple point mutant was eliminated in vivo, although tumor necrosis factor-inducible IκBα degradation was unaffected. In cell lines and in transiently transfected cells, mutation of the CKII sites in IκBα resulted in a protein with increased intrinsic stability. Together with results demonstrating a role for N-terminal sites in inducer-mediated phosphorylation and degradation of IκBα, these studies indicate that CKII sites in the C-terminal PEST domain are important for constitutive phosphorylation and intrinsic stability of IκBα.

NF-κB proteins are a family of pleiotropic transcription factors that regulate expression of numerous cytokines, and viral genes (for reviews, see references 27 and 48). In cells, NF-κB is present in an inactive cytoplasmic form; localization of the p50-p65 heterodimeric form of NF-κB to the cytoplasm is mediated by the inhibitory IκBα protein that binds to and masks the nuclear localization signal of the p65 subunit of NF-κB (5, 7). Activating agents, such as viruses, phorbol esters, cytokines, radical oxygen intermediates, and bacterial lipopolysaccharide, promote the dissociation of the cytosolic NF-κB/IκB complexes, in part by activating cellular kinases that phosphorylate IκB (5–7, 22, 27, 48).

The DNA-binding NF-κB family members share a Rel homology domain that is responsible for DNA binding, nuclear localization, and protein dimerization. DNA-binding members of NF-κB/Rel include p50 (NFKB1) (9, 23, 41), p65 (RelA) (52, 58), c-Rel (13, 24), p52 (NFKB2 or Lyt-10) (8, 51, 62), RelB (I-Rel) (59, 60), and dorsal (65). p50 and p52 are synthesized as precursors of p105 and p100, respectively, that are proteolytically processed to generate active DNA-binding p50 and p52 (8, 9, 23, 41, 51, 62). Different NF-κB dimers bind to variant NF-κB sites (44) (consensus, 5′-GGGANNYYCC-3′) present in the promoter regions of many genes (reviewed in references 2 and 48).

* Corresponding author. Mailing address: Lady Davis Institute for Medical Research, 3755 Côte Ste. Catherine, Montreal, Quebec H3T 1E2, Canada. Phone: (514) 340-8260, ext. 5265. Fax: (514) 340-7576.

The intracellular localization and posttranslational activity of NF-κB/Rel proteins are regulated by the ankyrin repeat-containing IκB proteins (IκBα, IκBγ, Bcl-3, p105, and p100) (reviewed in references 5, 25, 28, and 39). Phosphorylation by activated cellular kinases and subsequent degradation of IκBα appear to be critical regulatory steps in the control of NF-κB activation. Recent studies demonstrated that mutation of either Ser-32 or Ser-36 blocked signal-induced IκBα phosphorylation and degradation (10, 11, 69), and a truncated IκBα that was missing 45 amino acids at the N terminus corrected radiation sensitivity in ataxia telangiectasia cells (37). Furthermore, IκBα transcription is upregulated by NF-κB, since the IκBα promoter contains functional NF-κB sites. Thus, an autoregulatory loop is established, and the ultimately increased IκBα concentrations limit the nuclear translocation of NF-κB proteins (12, 18, 45, 66). In recent studies, disruption of IκBα autoregulation with IκBα antisense RNA resulted in malignant transformation and altered NF-κB-regulated gene activity, indicating that IκBα may represent a potential growth suppressor activity (4).

To further characterize the signaling events that contribute to IκBα phosphorylation and degradation, a kinase activity that specifically interacted with IκBα in an affinity chromatography step and phosphorylated IκBα with high specificity in vitro was isolated from Jurkat T cells. Biochemical and immunological cross-reactivity experiments identified the kinase activity as casein kinase II (CKII). Point mutation analysis revealed that a triple mutation of residues T-291, S-283, and T-299 eliminated phosphorylation of IκBα by the CKII in vitro

and constitutive phosphorylation of IκBα in vivo, although inducible IκBα degradation was unaffected. Together with results demonstrating a critical role for N-terminal sites in inducer-mediated phosphorylation and degradation of IκBα, these studies indicate that CKII sites in the C-terminal domain are important for constitutive phosphorylation and intrinsic stability of IκBα.

MATERIALS AND METHODS

Plasmid constructions. Plasmids for the expression of recombinant NF-κB subunits p50 and p65, p50-p65 chimeric protein, and wild-type IκBα–glutathione *S*-transferase (GST) fusion protein were described previously (21, 47). The plasmid encoding the polyhistidine-tagged IκBα protein was constructed by ligating the 1,190-bp *Eco*RI fragment (filled in with Klenow enzyme) from IκBα/pGEX-2T into the *Xho*I site (filled in with Klenow enzyme) of pET-15b (Novagen). Wild-type human IκBα (wtIκBα) or mutated human IκBα cDNA was inserted downstream of the simian virus 40 promoter in the pSVK3 vector (Pharmacia) for transient transfection.

Mutagenesis. cDNAs encoding IκBα carboxyl-terminal deletion mutations were generated by 25 cycles of PCR amplification. DNA oligonucleotide primers were synthesized with an Applied Biosystems DNA/RNA synthesizer. The amino-terminal primer was synthesized with an *Eco*RI restriction enzyme site, and the carboxyl-terminal primers were synthesized with *Eco*RV restriction enzyme sites at their ends. The PCR products were purified by phenol-chloroform extraction and ethanol precipitation, digested with *Eco*RI and *Eco*RV, and inserted into the *Eco*RI-*Tth*111I fragment (the *Tth*111I site was filled in with Klenow enzyme) of pGEX-2T (Pharmacia Biotech Inc.). The point mutations of IκBα were generated by overlap PCR mutagenesis with *Pfu* DNA polymerase (33). Mutations were confirmed by sequencing.

Expression and purification of recombinant proteins. GST fusion proteins from *Escherichia coli* and polyhistidine-tagged proteins from the baculovirus expression system were isolated as described previously (47). For the expression of polyhistidine-tagged IκBα protein, the plasmid IκBα/pET-15b was transformed into *E. coli* BL21(DE3) and induced by isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C.

In vitro protein kinase assay. Polyhistidine-tagged IκBα or p50-65 recombinant proteins (20 μg) were expressed and bound to His-Bind metal chelation resin prior to incubation with 10 mg of Jurkat whole-cell extracts at room temperature for 1 h. After three washes with binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.9]), the bound proteins were eluted with 200 μl of 6 M urea in binding buffer. The in vitro kinase assays were performed for 30 min at 30°C with 1 μl of eluted bound proteins or 5 U of recombinant CKII enzyme, 20 to 50 ng of substrate, and 5 μCi of [γ-^{32}P]ATP or [γ-^{32}P]GTP in a buffer containing 25 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM $MnCl_2$, and 1 mM $MgCl_2$. Reactions were stopped by the addition of 2× sample buffer; this was followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

In-gel kinase assay. In-gel kinase assays were performed as described by Hibi et al. (32) with slight modifications. Whole-cell extracts (100 μg) or partially affinity-purified kinase (10 μl) were resolved on an SDS–10% polyacrylamide gel polymerized in the presence of 10 μg of either GST or GST-IκBα per ml. After electrophoresis, the gel was washed twice for 30 min at room temperature with 100 ml of 20% isopropanol–50 mM Tris-HCl (pH 7.6) to remove SDS and then twice for 30 min at room temperature with 100 ml of buffer A (50 mM Tris-HCl [pH 7.6], 5 mM 2-mercaptoethanol). The gel was then incubated in 100 ml of 6 M urea in buffer A at room temperature for two 30-min incubations, followed by serial incubations in buffer A containing 0.05% Tween 20 and 3, 1.5, or 0.75 M urea, each for 30 min at room temperature. The gel was washed five times for 1 h each with 100 ml of buffer A containing 0.05% Tween 20 at 4°C and incubated in the same buffer at 4°C overnight. The gel was finally incubated in 50 ml of buffer C for 1 h at room temperature and then in 10 ml of buffer C plus 5 μCi of [γ-^{32}P]ATP per ml for 1 h at room temperature. The gel was washed with 100 ml of 5% trichloroacetic acid–1% sodium PP_i at room temperature several times, until the radioactivity in the solution was negligible; it was then dried and subjected to autoradiography.

Construction of IκBα-expressing cells. Isolation of NIH 3T3 cells expressing wtIκBα, IκBα(S283A, T291A) or IκBα(S283A, T291A, T299A) will be described in detail elsewhere (4a). Briefly, cDNA encoding the Tet^r-VP16 transactivator tTA (26) was inserted into the *Hin*dIII-*Bam*HI fragment of the hygromycin resistance plasmid pREP4 (Invitrogen), under control of the Rous sarcoma virus promoter. wtIκBα or mutant IκBα cDNAs were cloned downstream of the tetracycline responsive promoter CMV_t, which consists of a minimal promoter sequence derived from the human cytomegalovirus immediate-early (IE) promoter fused to seven copies of *tet* operator sequences (26). These CMV_t-IκBα cassettes were inserted into the neomycin (G418) resistance plasmid pREP9 (Invitrogen) between the *Xho*I and *Bam*HI sites, thus replacing the Rous sarcoma virus promoter. Plasmids pREP4-tTA and pREP9-CMV_t-IκBα were sequentially introduced into NIH 3T3 cells by transfection (Lipofectamine; Gibco BRL) as specified by the manufacturer. At 48 h after transfection, cells were selected in Dulbecco's modified Eagle's medium containing 10% calf serum, 300 μg of hygromycin per ml, and 400 μg of G418 per ml. Colonies of drug-resistant cells were expanded and tested for expression of wtIκBα or mutant IκBα when cultured in the presence or absence of tetracycline.

Immunoblot analysis of IκBα turnover. NIH 3T3 cells were stably or transiently transfected with wtIκBα or mutated IκBα-expressing plasmids, cultured in medium supplemented with 10% calf serum and treated with 50 μg of cycloheximide per ml in the presence or absence of 5 ng of tumor necrosis factor alpha (TNF-α; Gibco-BRL) per ml. Cells were washed with phosphate-buffered saline (PBS) and lysed in WBL buffer (10 mM Tris HCl [pH 8.0], 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 0.01 μg of leupeptin per μl, 0.01 μg of pepstatin per μl, 0.01 μg of aprotinin per μl). Equivalent amounts of protein (15 μg) were electrophoresed on a gradient SDS–7 to 13% polyacrylamide gel. Proteins were transferred to nitrocellulose, and IκBα was detected with affinity-purified IκBα antibody AR20 (55), an antipeptide antibody directed against the N-terminal amino acids (aa) 2 to 16. A monoclonal antibody, kindly provided by R. T. Hay, directed against aa 21 to 48 was also used in some experiments.

Detection of phosphorylated IκBα in vivo. For immunoprecipitation of $^{32}P_i$-labeled (or [^{35}S]methionine-labeled) proteins, NIH 3T3 cells expressing wtIκBα or mutant IκBα proteins were washed twice with Dulbecco's modified Eagle's medium without serum and phosphate (or without methionine) and then incubated for 45 min in Dulbecco's modified Eagle's medium containing 2.5% dialyzed calf serum; the medium was then replaced with fresh Dulbecco's modified Eagle's medium supplemented with 10% dialyzed calf serum and 1 mCi of $^{32}P_i$ per ml (or 300 μCi of [^{35}S]methionine per ml). The cells were labeled at 37°C with shaking for 6 h (4 h for [^{35}S]methionine). For TNF-α induction, cells were pretreated with 100 μM calpain inhibitor I for 1 h and then stimulated with TNF-α (5 ng/ml) for 30 min. The labeled cells were washed with PBS and lysed in TNT buffer (20 mM Tris HCl [pH 7.5], 200 mM NaCl, 0.5% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride). Cell lysates (300 μg) were incubated with 10 μl of IκBα antibody (AR20) and 30 μl of protein A-Sepharose beads (Pharmacia) at 4°C overnight. The beads were washed five times with TNT buffer, and the immunoprecipitate was eluted by boiling the beads for 3 min in 30 μl of 0.5% 2-mercaptoethanol–1% SDS. The eluate was then diluted 1:2 with TNT buffer and incubated with 10 μl of IκBα antibody and 30 μl of protein A-Sepharose beads for 6 h at 4°C. The beads were again washed five times with TNT buffer, and the immunoprecipitate was eluted by boiling the beads for 3 min in SDS sample buffer. Eluted proteins were electrophoresed by SDS-PAGE (10% polyacrylamide). Proteins were transferred to nitrocellulose and detected by autoradiography.

Phosphoamino acid analysis. Recombinant IκBα proteins were phosphorylated in vitro with [γ-^{32}P]ATP as described above. The proteins were separated on a 10% acrylamide gel and transferred to a polyvinylidene difluoride membrane. The labeled bands corresponding to IκBα were excised and subjected to acid hydrolysis in 5.7 M HCl for 1 h at 110°C (38). The phosphoamino acids were separated by one-dimensional thin-layer chromatography with an optimized pH 2.5 buffer (35). The plates were exposed to phosphoscreen and analyzed with a PhosphorImager apparatus (Molecular Dynamics).

RESULTS

Detection of a protein kinase binding to IκBα. To identify the protein kinase(s) involved in phosphorylation of IκBα, the possibility that an IκBα-specific kinase is physically interacting with IκBα was investigated. IκBα was expressed as a polyhistidine-tagged protein in *E. coli*; as a control, the His-tagged p50-65 fusion protein expressed in baculovirus was used (47). Both expressed proteins were bound to His-Bind metal chelation resin and incubated with whole-cell extract derived from Jurkat T cells. After washing of the column and elution with 6 M urea, in vitro kinase assays were performed to examine whether any of the IκBα-binding proteins possessed IκBα-specific kinase activity. With rIκBα as a substrate, a kinase was identified in the bound proteins eluted from the IκBα affinity column (Fig. 1, lane 4); a small amount of kinase was also eluted from the p50-65 affinity column (lane 5). The absence of kinase in the p50-65 lane was not due to the lack of substrate, since an equal amount of IκBα protein was added in each lane. Previous studies had demonstrated that a serine kinase was associated with the NF-κB/IκBα complex and phosphorylated p50 and p65 subunits but not IκBα (30, 53). To investigate whether the kinase activity associated with IκBα phosphorylated other NF-κB subunits, recombinant p50, p65, or the p50-65 chimeric protein was used as the substrate in the kinase

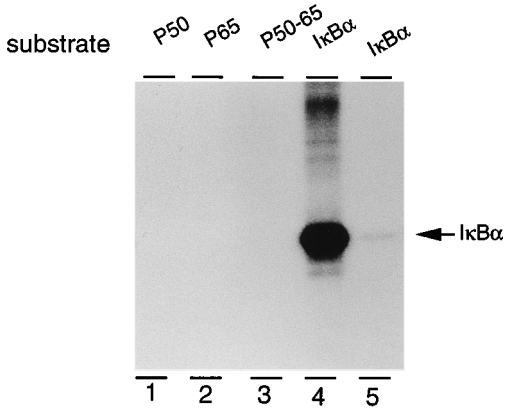

FIG. 1. A kinase interacts with IκBα protein. Polyhistidine-tagged IκBα (lanes 1 to 4) or p50-65 (lane 5) recombinant proteins were expressed and bound to His-Bind metal chelation resin. The His-Bind resins were incubated with Jurkat whole-cell extracts at room temperature for 1 h. After three washes with binding buffer A, the bound proteins were eluted with 6 M urea in binding buffer. The eluted proteins were analyzed by in vitro kinase assays under standard phosphorylation conditions. Recombinant p50 (lane 1), p65 (lane 2), p50-65 (lane 3), and IκBα (lanes 4 and 5) were used as substrates (all at ~50 ng). Phosphorylation was analyzed by SDS-PAGE followed by autoradiography.

assays; as shown in Fig. 1, lanes 1 to 3, these proteins were not phosphorylated by the IκBα-specific kinase.

The apparent mass of the kinase isolated from the IκBα affinity chromatography step was assessed by an in-gel kinase assay, with GST or GST-IκBα as the substrate. In experiments performed in the presence of GST, no phosphorylated protein was observed (Fig. 2, lanes 1 to 4) and autophosphorylation of the kinase was not detected in the absence of an exogenous substrate. However, when recombinant IκBα (IκBα-GST) was used as the substrate, two kinases migrating at 43 and 38 kDa were detected in either Jurkat whole-cell extracts (lanes 5 to 7) or proteins eluted from the IκBα affinity chromatography step (lane 8). The kinases were not affected by inducers of NF-κB DNA-binding activity such as TNF-α, anti-CD3 antibody, poly(rI-rC), or phorbol 12-myristate 13-acetate suggesting a constitutive kinase activity (lanes 6 and 7) (data not shown).

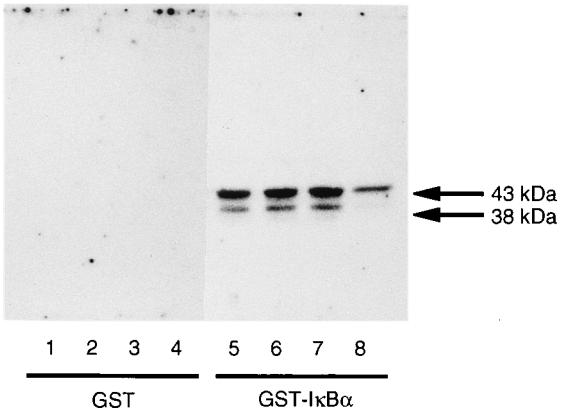

FIG. 2. Analysis of IκBα-associated kinase activity by in-gel kinase assay. IκBα-specific protein kinase activity was measured after SDS-PAGE by using an in-gel kinase assay with the GST (control, lanes 1 to 4) or GST-IκBα (substrate, lanes 5 to 8) (10 μg/ml) polymerized into the gel. Whole-cell extracts (100 μg) from uninduced (lanes 1 and 5), TNF-α-induced (10 ng/ml, treated for 5 min [lanes 2 and 6]), or phorbol 12-myristate 13-acetate-induced (40 ng/ml, treated for 15 min [lanes 3 and 7]) cells or 10 μl of bound proteins eluted from the IκBα affinity column were separated by SDS-PAGE and analyzed by the in-gel kinase assay (described in Materials and Methods).

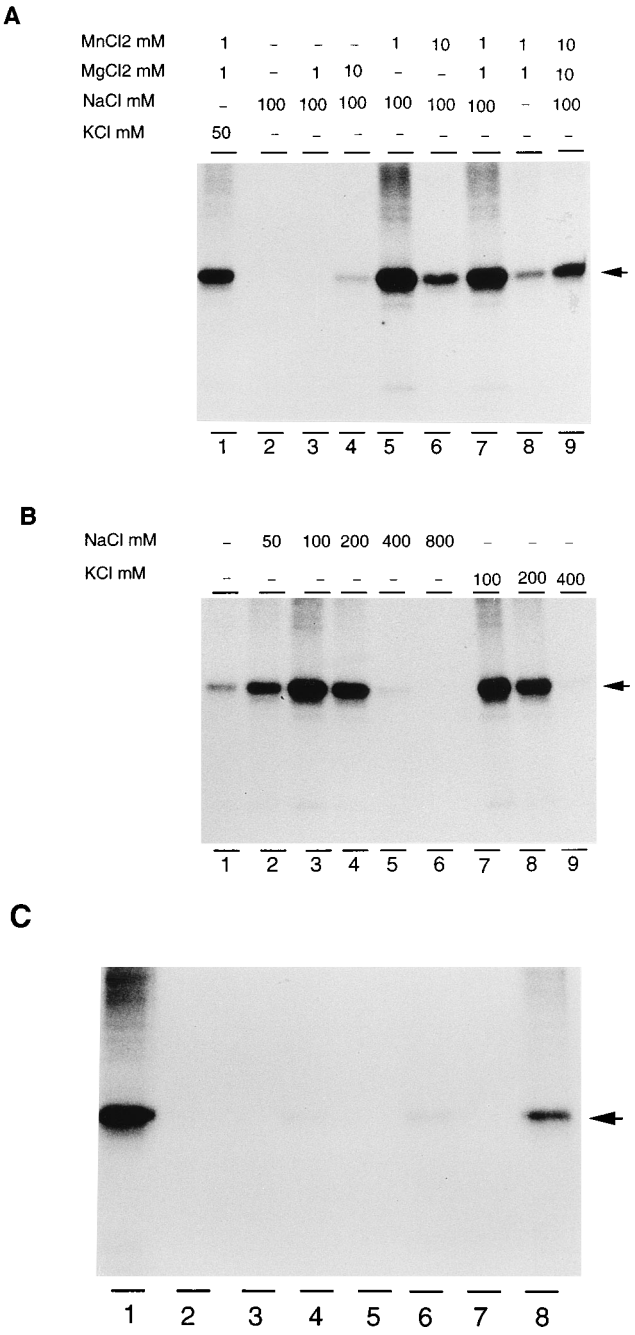

FIG. 3. Biochemical characterization of the IκBα-associated kinase activity. (A) Divalent-cation requirement for kinase activity. In vitro kinase reactions were performed in a buffer containing various divalent cations as indicated and IκBα as the substrate. (B) Na^+ and K^+ stimulate kinase activity. In vitro kinase reactions were carried out as in panel A, except that the buffer contained 1 mM each Mn^{2+} and Mg^{2+} and the indicated amount of NaCl or KCl. (C) Kinase reactions in the presence of CKII effectors. Kinase assays were performed under standard conditions (lane 1) or in the presence of CKII effectors as indicated: 0.1 mM ATP (lane 2), 1 mM ATP (lane 3), 0.1 mM GTP (lane 4), 1 mM GTP (lane 5), 10 μg of heparin per ml (lane 6), or 100 μg of heparin per ml (lane 7). In lane 8, the [γ-^{32}P]ATP was replaced by [γ-^{32}P]GTP.

Characterization of the IκBα-associated kinase. To characterize the kinase further, divalent-cation requirements and salt requirements were examined. Either Mg^{2+} or Mn^{2+} was necessary for kinase activity, but there was a preference for Mn^{2+} (Fig. 3A, lanes 4 and 5); Ca^{2+} and Zn^{2+} did not stimulate

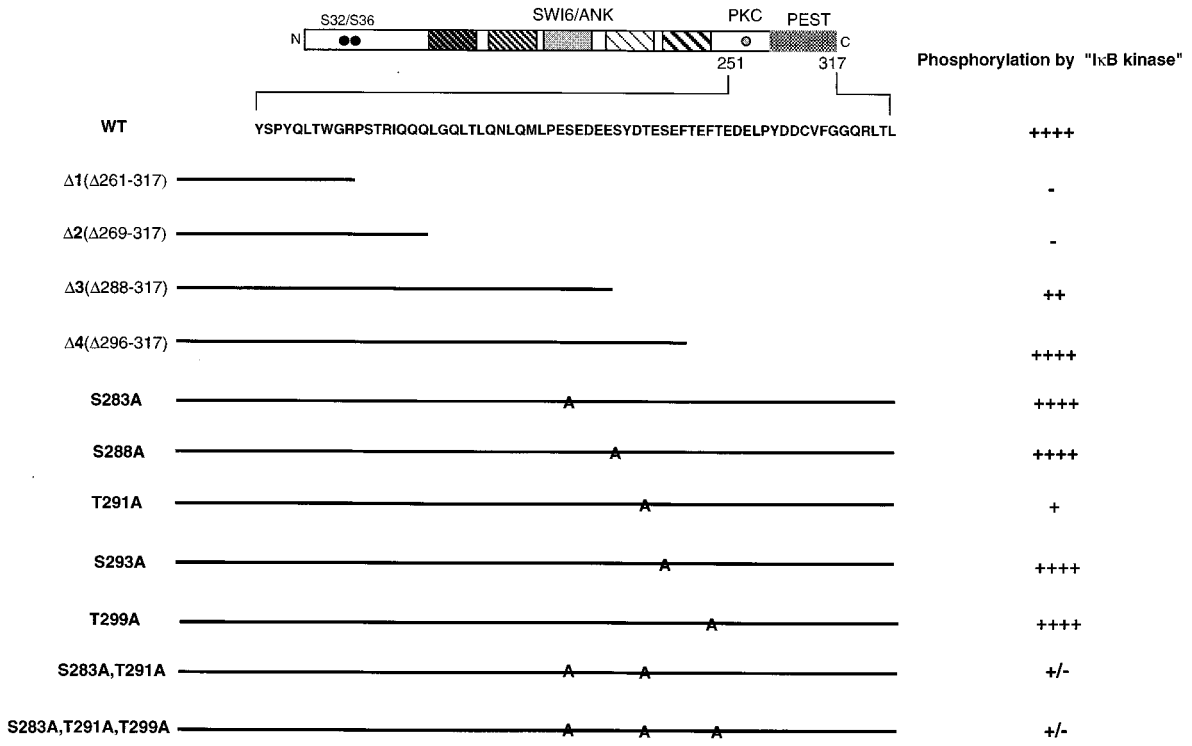

FIG. 4. Phosphorylation of IκBα mutants. The 317 amino acids of the human IκBα protein are illustrated schematically. The hatched boxes represent the ankyrin repeat domain; the open box represents the N-terminal domain (aa 1 to 73), and the shaded box represents the C-terminal PEST-like domain (aa 281 to 317). The amino acid sequence of the C-terminal region is shown from residues 261 to 317. The endpoints of the deletions are indicated by the solid line. For point mutations, the substituted alanine residues are indicated. Point mutations are described on the left in one-letter code as wild-type (WT) amino acid, the residue number, and the mutant amino acid. Phosphorylation in vitro by the IκBα-specific kinase is summarized on the right.

kinase activity (data not shown). In addition, kinase activity was stimulated by 50 to 200 mM NaCl (Fig. 3B, lanes 2 to 4). Similarly, replacing NaCl by KCl also simulated kinase activity (lanes 7 and 8). However, a high concentration of NaCl or KCl (>400 mM) inhibited kinase activity (lanes 5, 6, and 9).

The apparent mass of the kinase from the in-gel kinase assays was similar to the molecular masses of CKIIα (CKIIα and CKIIα′). CKII is a serine/threonine kinase that phosphorylates acidic substrates, utilizes Mg^{2+} and Mn^{2+} as divalent cations, utilizes GTP efficiently as a phosphate donor, and is inhibited by heparin addition (29, 50). The kinase assays were repeated in the presence of these CKII effectors. Phosphorylation in vitro by the IκBα-associated kinase was inhibited by unlabeled GTP (Fig. 3C, lanes 4 and 5), and the [γ-^{32}P]ATP could be replaced by [γ-^{32}P]GTP (lane 8), indicating that both ATP and GTP were utilized as phosphate donors. Furthermore, the kinase activity was totally inhibited by heparin at 100 μg/ml (lane 7). These biochemical properties strongly suggested that the IκBα-specific kinase identified by IκBα affinity chromatography was CKII.

Mapping the phosphorylation sites in IκBα that are phosphorylated by the associated kinase. To identify the region of IκBα specifically phosphorylated by the IκBα-associated kinase, a series of C-terminal deletions that generated IκBα proteins of 260 aa (Δ1), 268 aa (Δ2), 287 aa (Δ3), and 295 aa (Δ4) were constructed (Fig. 4). The truncated IκBα proteins were used as the substrate for in vitro protein phosphorylation assays with kinase partially purified by affinity chromatography. Results of these experiments suggested that the phosphorylation sites were located in the C-terminal PEST-like sequence (Fig. 4). In IκBαΔ1 and IκBαΔ2, no phosphorylation was detected. IκBαΔ4 was phosphorylated as efficiently as the wild-type IκBα protein, whereas IκBαΔ3 was phosphorylated with lower efficiency. Analysis of these deletions suggested that more than one phosphorylation site was present in the PEST-like sequence of IκBα, with at least one site located between residues 269 and 287 and another between residues 288 and 295.

Serine and threonine residues within the region spanning residues 269 to 295 were specifically mutated to alanine to identify critical phosphoamino acid residues. Mutations in Ser-283 (S283A), Ser-288 (S288A), or Ser-293 (S293A) had little or no effect on the in vitro phosphorylation of IκBα by its associated kinase (Fig. 5, lanes 3, 4, and 6), whereas mutation in Thr-291 (T291A) reduced the phosphorylation by two- to four-fold (lane 5). However, the Ser-283, Thr-291 double mutation (S283A, T291A) or the S283A, T291A, T299A triple mutation reduced phosphorylation up to 20-fold (lanes 7 and 8). Importantly, Ser-283, Thr-291, and Thr-299 all lie within a consensus CKII phosphorylation site (S/T-X-X-E/D).

To determine whether the IκBα-associated kinase was indeed CKII, recombinant CKII was used to phosphorylate wild-type or mutated IκBα proteins (Fig. 5C and D). The results demonstrated clearly that recombinant CKII produced similar phosphorylation results with wild-type and mutant proteins to those obtained with the IκBα-associated kinase. Finally, an immunodepletion experiment was performed with anti-CKII antibody. Proteins bound to His-tagged IκBα were eluted, incubated with antibody specific for CKIIα, and precipitated with protein A-Sepharose. The supernatant was saved and used in the in vitro phosphorylation assay with rIκBα as a substrate (Fig. 6A) and in immunoblot analysis with antibody to CKIIα (Fig. 6B). The partially purified proteins without treatment or incubated with preimmune serum phosphorylated IκBα (Fig. 6A, lanes 1 and 3) and contained CKIIα (Fig. 6B,

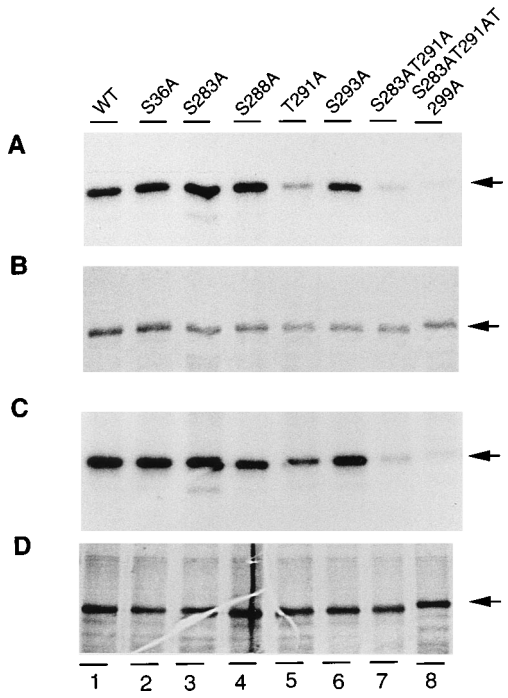

FIG. 5. Mapping phosphorylation sites in IκBα protein. Wild-type and point mutants of IκBα (as indicated above the lanes) were purified as described previously (47). These proteins (~50 ng each) were used as substrates for in vitro kinase reactions with either affinity-purified IκBα-associated kinase (A and B) or recombinant CKII (C and D). The proteins were silver stained (B and D) before autoradiography (A and C).

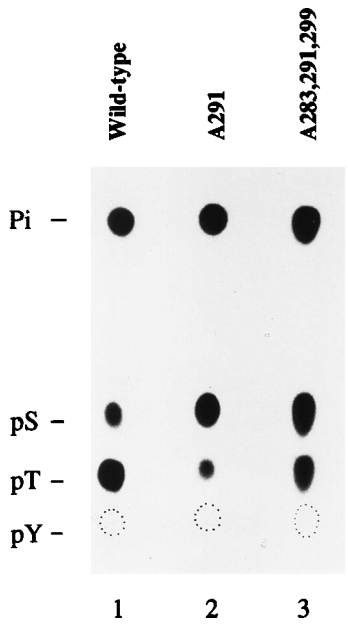

FIG. 7. Phosphoamino acid analysis of the in vitro ^{32}P-labeled IκBα proteins. The recombinant wtIκBα (lane 1), IκBα (T291A) (lane 2), and IκBα(S283A, T291A, T299A) (lane 3) proteins were phosphorylated in vitro by bound proteins eluted from the IκBα affinity column. The phosphorylated amino acids were separated by one-dimensional electrophoresis on thin-layer cellulose plates. Abbreviations: pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine.

lanes 1 and 3), whereas after immunodepletion, there was no detectable kinase activity (Fig. 6A, lane 2) and no detectable CKIIα subunit by immunoblot (Fig. 6B, lane 2). Taken together, the data demonstrate that the IκBα-associated kinase has all the characteristics of the catalytic subunit of CKIIα.

Phosphoamino acid analysis of wtIκBα and mutated IκBα. To further characterize the effect of point mutations on the phosphorylation state of IκBα, the target amino acid residues on wtIκBα, IκBα(T291A), and IκBα(S283A, T291A, T299A; also referred to as IκB-3C) were determined by phosphoamino acid analysis (15). Equal amounts of the three proteins were phosphorylated in vitro by the IκBα-associated kinase; the counts per minute (cpm) incorporated into IκBα(T291A) protein was reduced twofold compared with wtIκBα, while the cpm incorporated into IκBα-3C was 20-fold lower than that for wtIκBα (see Fig. 5). Phosphoamino acid analysis of IκBα proteins (equal cpm) demonstrated that both serine and threonine residues were phosphorylated by the kinase (Fig. 7). Strikingly, the ratio of phosphothreonine to phosphoserine was 4:1 with wtIκBα but was reversed (1:4) with the IκBα(T291A) mutant. Similarly, with IκBα-3C, the pT/pS ratio was reversed (1:2) compared with wtIκBα.

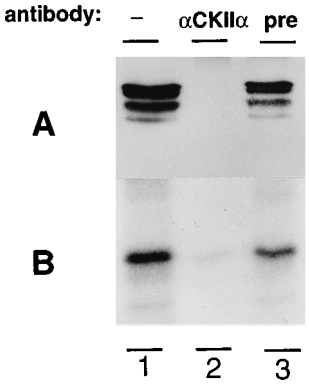

FIG. 6. Immunodepletion of IκBα-associated kinase activity by CKIIα antibody. The proteins eluted from the IκBα affinity column (100 μl) were incubated with 10 μl of CKIIα antibody (lane 2) or normal serum (lane 3) and precipitated with protein A-Sepharose (30 μl) at 4°C for 30 min. Supernatant material (10 μl) or eluted proteins before immunoprecipitation (lane 1) were used for the in vitro kinase assay with wtIκBα protein (~50 ng) as the substrate (B) and in immunoblotting analysis with CKIIα antibody (A).

Detection of phosphorylated IκBα in vivo. In vivo, IκBα is constitutively phosphorylated in addition to its inducible phosphorylation (11, 17). To investigate whether the C-terminal CKII sites were required for IκBα constitutive phosphorylation, stably transfected cells expressing wtIκBα, IκBα-3C, or control NIH 3T3 tTA cells were labeled in vivo with $^{32}P_i$ or [^{35}S]methionine. The IκBα proteins were purified by double immunoprecipitation, separated by SDS-PAGE and detected by autoradiography (Fig. 8). Endogenous murine IκBα was detected as a constitutively phosphorylated protein in all samples (Fig. 8A, lanes 1 to 6), human wtIκBα was detected in lanes 3 and 4, while the phosphorylation of IκBα-3C was undetectable (lanes 5 and 6). Treatment with TNF in the presence of calpain inhibitor I for 30 min also failed to reveal any phosphorylation of the IκBα-3C. TNF-mediated phosphorylation of human wtIκBα was detectable with dark exposure of the autoradiograph (data not shown). The failure to detect phosphorylation in IκBα-3C-expressing cells was not due to the absence of protein, since expression of IκBα-3C was detectable by immunoblotting from the same membrane used in Fig. 8A with monoclonal human IκBα antibody MAD 10B (34) (Fig. 8B, lanes 5 and 6). Furthermore, the expression of IκBα-3C was also detectable when proteins were labeled with

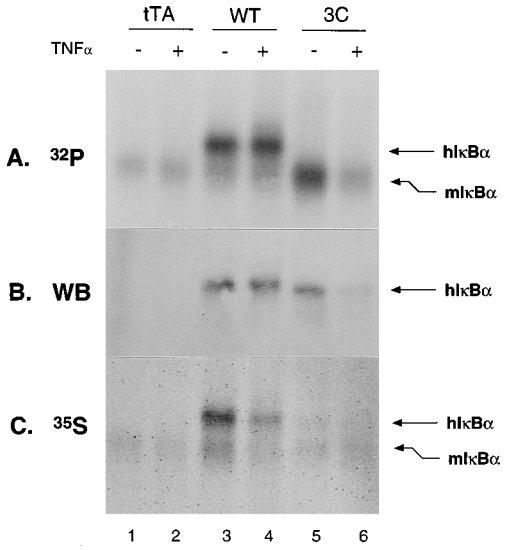

FIG. 8. Constitutive phosphorylation in IκBα-expressing NIH 3T3 cells. (A and C) Stably transfected NIH 3T3 tTA- (lanes 1 and 2), wtIκBα- (lanes 3 and 4) (wt), and IκBα-3C (lanes 5 and 6)-expressing cells were labeled with $^{32}P_i$ (A) or [^{35}S]methionine (C), untreated (lanes 1, 3, and 5) or treated with calpain inhibitor I (100 μM) and TNF-α (5 ng/ml) (lanes 2, 4, and 6). Extracts (300 μg) were then double immunoprecipitated with IκBα polyclonal antibody (AR20), and the immunoprecipitates were subjected to SDS-PAGE, transferred to a membrane, and autoradiographed. (B) The expression of human IκBα in $^{32}P_i$-labeled cells was detected by Western blot (WB) from the same membrane as used in panel A, with the monoclonal human IκBα antibody directed against aa 21 to 48 (34).

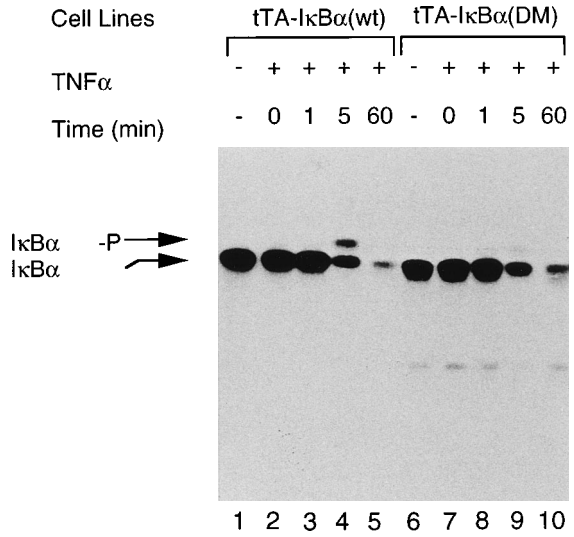

FIG. 9. TNF-α-induced phosphorylation in IκBα-expressing NIH 3T3 cells. Protein extracts (50 μg) from wtIκBα- and IκBα(S283A, T291A) [termed IκBα(DM)]-expressing cells were electrophoresed on an SDS–7 to 13% gradient polyacrylamide gel, transferred to nitrocellulose, and analyzed with IκBα antibody AR20. Hyperphosphorylated IκBα (IκBα-P) appears as a distinct, more slowly migrating protein band. wtIκBα cells (lanes 1 to 5) and IκBα(S283A, T291A) cells (lanes 6 to 10) were either left untreated (lanes 1 and 6) or stimulated with 5 ng of TNF-α per ml for 10 s (lanes 2 and 7), 1 min (lanes 3 and 8), 5 min (lanes 4 and 9), or 60 min (lanes 5 and 10).

[^{35}S]methionine and purified by double immunoprecipitation (Fig. 8C, lanes 5 and 6).

NIH 3T3 cells expressing wtIκBα or IκBα(S283A, T291A) under the control of the Tet-responsive promoter were used to determine if the C-terminal mutations of IκBα altered phosphorylation and/or degradation of IκBα in vivo. NIH 3T3 cells were stimulated with TNF-α (5 ng/ml) for 1, 5, or 60 min (Fig. 9); hyperphosphorylation of wtIκBα was detected by the appearance of a more slowly migrating IκBα band within 1 or 5 min of TNF treatment (Fig. 9, lanes 3 and 4), as described previously (6, 68). The slowly migrating form of IκBα decreased when extracts were treated with CIP but not when phosphatase inhibitors were included in the CIP reaction (data not shown), thus confirming the phosphorylated nature of IκBα. No phosphorylated IκBα was detected at 60 min after TNF-α treatment (lane 5), reflecting the rapid and transient nature of IκBα phosphorylation (6, 68). The double-mutant IκBα(S283A, T291A)—and in independent experiments IκBα-3C—was only weakly phosphorylated compared with wtIκBα at 5 min after TNF-α treatment (lane 9); however, both wtIκBα and IκBα(S283A, T291A) degraded to approximately the same extent by 60 min after TNF-α treatment (lanes 5 and 10). These experiments suggest that mutations in the C-terminal CKII sites affect the overall level of IκBα phosphorylation but do not block IκBα degradation in vivo, thus complementing the results of Brown et al. (11) demonstrating the requirement for phosphorylation at Ser-32 and Ser-36.

Mutated C-terminal CKII sites increase IκBα intrinsic stability. To examine the effect of point mutations on the degradation of IκBα, the turnover rate of IκBα was measured in cells stably expressing IκBα and treated with TNF-α in the presence of the protein synthesis inhibitor cycloheximide at different times after TNF-α addition (Fig. 10). Following TNF-α stimulation, both wtIκBα and IκBα-3C degraded rapidly (Fig. 10A and B, lanes 7 to 12). In contrast, independent experiments confirmed that IκBα(S36A) was resistant to TNF-induced degradation (data not shown). Therefore, the triple point mutation in IκBα-3C did not alter inducer-mediated degradation. However, in the presence of cycloheximide but without inducer, IκBα-3C appeared more stable (Fig. 10B, lanes 1 to 6) than wild-type protein (Fig. 10A, lanes 1 to 6). To confirm that the mutation in the C-terminal sites affected the intrinsic stability of IκBα protein, NIH 3T3 cells were also transiently transfected with wtIκBα or IκBα-3C, and IκBα turnover in the presence of cycloheximide was measured. As shown in Fig. 10C and D, the half-life of IκBα-3C was about 90 min compared with 30 min for wtIκBα, demonstrating that mutation of CKII phosphorylation sites increased IκBα intrinsic stability but did not affect inducer-mediated degradation.

DISCUSSION

In this report, we describe the characterization of a protein kinase activity isolated from Jurkat T cells that specifically interacted with and phosphorylated recombinant IκBα. Deletion analysis of IκBα localized the phosphorylation sites to the C-terminal PEST-like domain. Point mutation analysis revealed that double mutation of residues T-291 and S-283 dramatically reduced phosphorylation by the kinase both in vitro and in vivo. This protein kinase was identified as CKII on the basis of the following biochemical and immunological criteria. (i) The kinase preferred Mn^{2+} and Mg^{2+} as divalent cations, was stimulated by Na^+ and K^+, was inhibited by heparin, and utilized ATP or GTP as a phosphate donor. All of these characteristics are consistent with CKII. (ii) The bound proteins eluting from the IκBα affinity column contained the CKII catalytic subunit as detected by immunoblotting with CKIIα antibody, and immunodepletion of the extract with CKIIα antibody eliminated the IκBα-specific kinase activity. (iii) The patterns of phosphorylation of the single, double, and triple

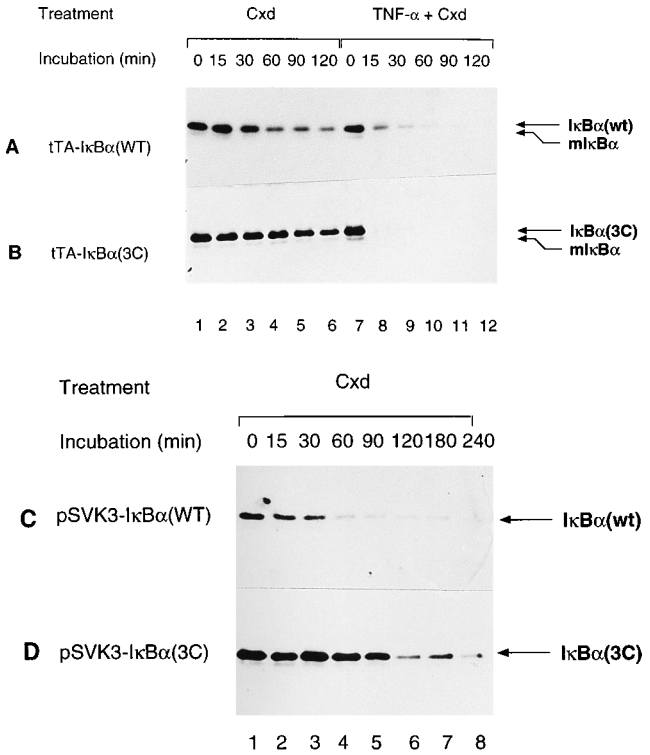

FIG. 10. Analysis of IκBα turnover rate. (A and B) NIH 3T3 cells stably transfected with tTA-wtIκBα (A) and tTA-IκBα-3C (B) were treated with cycloheximide (Cxd) (50 μg/ml) alone (lanes 1 to 6) or stimulated with TNF-α (5 ng/ml) in the presence of cycloheximide (lanes 7 to 12) for 0 (lanes 1 and 7), 15 (lanes 2 and 8), 30 (lanes 3 and 9), 60 (lanes 4 and 10), 90 (lanes 5 and 11), or 120 (lanes 6 and 12) min. IκBα was detected in whole-cell extracts (15 μg) by immunoblotting with AR20 antibody. (C and D) NIH 3T3 cells transiently transfected with wtIκBα (C) and IκBα-3C (D) (in pSVK3 vector) were treated with cycloheximide (50 μg/ml) for 0 (lane 1), 15 (lane 2), 30 (lane 3), 60 (lane 4), 90 (lane 5), 120 (lane 6), 180 (lane 7), or 240 (lane 8) min. IκBα was detected in whole-cell extracts (15 μg) by immunoblotting with AR20 antibody.

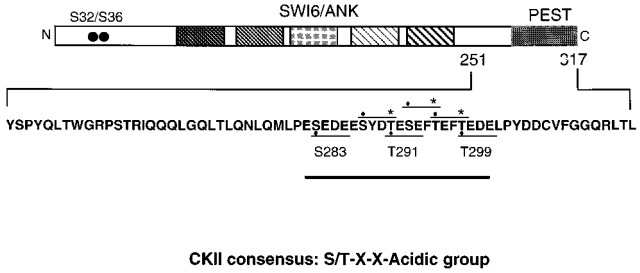

FIG. 11. Schematic representation of human IκBα and C-terminal CKII phosphorylation sites. Human IκBα contains five internal ankyrin repeats (SWI6/ANK) involved in the binding to NF-κB molecules. At the N-terminal of IκBα are two phosphorylation sites (S32 and S36), shown previously to play a role in inducer-mediated degradation (10, 11), indicated by the solid circles. A region rich in proline, serine, threonine, and glutamic acid, the PEST domain, spans aa 264 to 317; the C-terminal region of IκBα between aa 251 and 317 is expanded below the schematic to show the one-letter amino acid sequence. The PEST domain contains several CKII phosphorylation sites clustered between aa 283 and 302 at S-283, T-291 and T-299 (underlined). Phosphorylation of residues T-291 and T-299 also generates new potential CKII phosphorylation sites at residues S-288, T-296, and S-293 (denoted by lines and asterisks above the sequence).

point mutants by recombinant CKII and the IκBα-specific kinase were identical; in particular, mutation of the consensus CKII sites in IκBα(T291A, S283A, T299A) reduced phosphorylation by both the IκBα-associated kinase and recombinant CKII up to 20-fold. Furthermore, recombinant CKII also phosphorylated wild-type IκBα protein but not the C-terminal deletion mutant Δ1 protein (data not shown).

Mapping CKII phosphorylation sites in IκBα. CKII is a tetrameric serine/threonine-specific protein kinase composed of two large catalytic subunits (α and/or α′; relative molecular mass, 35 to 44 kDa) and two small β regulatory subunits (relative molecular mass, 25 to 28 kDa) (49). Immunocytochemical studies have localized CKII to the nucleus, the cytoplasm, and the plasma membrane (42, 70). CKII was demonstrated to phosphorylate a variety of nuclear factors including c-ErbA, adenovirus E1A, human papillomavirus E7, c-Myb, Fos, CREB/ATF, p53, SRF, and c-Jun (46, 49). Also, it was shown that CKII associated with HSP90 (64), FKBP25 (36), and p53 (19, 31, 50).

There are several potential sites of CKII serine/threonine phosphorylation in IκBα (consensus S/T-X-X-E/D), including residues S-32, S-36, S-283, T-291, and T-299. Interestingly, the glutamic or aspartic acid residues at position 4 of the consensus sequence may be substituted by any phosphoamino acid. Thus, residues S-288, T-296 and S-293 can also serve as potential CKII phosphorylation sites when residues T-291, T-299, or T-296, respectively, are phosphorylated. This possibility is illustrated schematically in Fig. 11. Deletion analysis of IκBα located the in vitro phosphorylation sites to the C-terminal PEST-like domain. Two critical sites (residues T-291 and S-283) were further identified by point mutation analysis. The fact that the T291A mutation reduced phosphorylation by two- to fourfold while other single point mutations had little or no effect suggested that residue T-291 was a critical phosphorylation site for CKII. The results of phosphoamino acid analysis in vitro supported this conclusion. In wtIκBα protein, the phosphothreonine/phosphoserine ratio was 4:1, while in IκBα (T291A), the ratio was reversed at 1:4. When S-283 alone was changed to alanine, there was no effect on the phosphorylation by CKII in vitro; however, the double mutation (T291A, S283A) reduced phosphorylation more than 10- to 20-fold. Combined with the fact that the T291A mutation only reduced phosphorylation by two- to fourfold, the results indicate that residue 283 is also an important site for CKII phosphorylation. It is possible that threonine 291 and/or serine 283 is responsible for positioning the kinase such that ordered series of phosphorylation events can occur, perhaps with T291 serving as an initial binding site for CKII. At present, the in vivo pattern of phosphorylation of S291A- or S283A-expressing cells is being examined.

CKII phosphorylates IκBα both in vitro and in vivo. Many protein kinases can phosphorylate IκB family proteins and activate NF-κB/IκBα complexes in cell-free systems (1). These kinases include protein kinase C (22), cyclic AMP-dependent protein kinase (40), heme-regulated eIF-2 kinase (22), Raf-1 kinase (20), and RNA-dependent protein kinase (43). Phosphorylation of IκBα in intact cells was detected following treatment of cells with TNF-α, lipopolysaccharide, or phorbol 12-myristate 13-acetate (6, 12, 16, 67). However, none of the kinases identified to date has been shown to directly phosphorylate IκBα in vivo. In this study, we identified in vivo sites of phosphorylation in IκBα located in the C-terminal PEST-like domain. It was previously shown that IκBα was constitutively phosphorylated in unstimulated cells and became hyperphosphorylated upon cell stimulation (11, 17). Here we show that the constitutive phosphorylation of IκBα is eliminated by mutation at residues T-291, S-283 and T-299 (IκBα-3C). Mutation of specific serine residues in the C-terminal PEST domain of the avian pp40 protein also eliminated phosphorylation of pp40 in vivo (61). Since the IκBα-3C mutation also eliminated

phosphorylation of IκBα by recombinant CKII in vitro, CKII appears to be the only protein kinase identified to date that can directly phosphorylate IκBα in vivo. After stimulation of cells with TNF-α, the hyperphosphorylated form of wtIκBα was detected by immunoblotting (Fig. 9); however, phosphorylation of IκBα(S283A, T291A) was reduced about 10-fold (Fig. 9), suggesting that an intact C-terminal domain may also contribute to the generation of the hyperphosphorylated form of IκBα.

Phosphorylation of IκBα by CKII alters its intrinsic stability. Since many eukaryotic proteins with a short half-life have PEST sequences and are phosphorylated by CKII, it has been suggested that phosphorylation may affect protein turnover (49). In both stably transfected cells and transiently transfected cells, the half-life of IκBα in the presence of cycloheximide was increased by the three CKII site mutations. In stably transfected cells, the half-life of wtIκBα was about 1 h, compared with 2 h for the 3C mutant, whereas in transiently transfected cells, the half life was about 30 min for wtIκBα compared with 90 min for the 3C mutant. However, like wtIκBα, IκBα-3C rapidly degraded in response to TNF-α induction, even though phosphorylation of mutated protein was undetectable in vivo. These results indicate that IκBα phosphorylation by CKII may not be required for inducer-mediated degradation but, rather, plays a role in the constitutive turnover of the protein.

CKII is a highly conserved and ubiquitous protein kinase. More than 50 proteins are known to be phosphorylated by CKII. Furthermore, CKII activity is unusually high in transformed and proliferating cells and tissues; a rapid, transient increase in the level of CKII occurs in a variety of cells exposed to mitogenic stimuli or to differentiation inducing signals (56). Studies with *Saccharomyces cerevisiae* demonstrated that the catalytic subunit of CKII is required for yeast cell growth (54). Conversely, phosphorylation of serine residues in c-Myb, c-ErbA, and c-fos by CKII may repress transcription of genes involved in metabolic regulation, cell growth, or differentiation. Mutation of CKII phosphorylation sites in these proteins was associated with their conversion to oncogenic proteins, whereas mutation of CKII phosphorylation sites in the adenovirus E1A protein converted this oncogene to a transformation-defective protein (reviewed in reference 49).

Recently, CKIIα transgenic mice that developed lymphoproliferative disorders resembling the fatal lymphoproliferative syndrome associated with the protozoan parasite *Theileria parva* in cattle were created (63). Interestingly, theileriosis in cattle is characterized by the overexpression of CKII. In the murine transgenic model, CKIIα-expressing mice displayed a stochastic potential to develop lymphoma; coexpression of a c-*myc* transgene in addition to CKIIα resulted in neonatal leukemia. Based on this study, CKII can serve as an oncogene, and its dysregulated expression is capable of transforming lymphocytes in cooperation with c-*myc* (63).

IκBα phosphorylation by CKII is not directly linked with inducer-mediated degradation. Recent studies demonstrated that the amino terminus of IκBα represents a signal response domain for activation of NF-κB. Substitution of alanine for either Ser-32 or Ser-36 completely abolished the signal-induced phosphorylation and degradation of IκBα and blocked the activation of NF-κB (10, 11, 69). These mutations also blocked in vitro ubiquitination of the IκBα protein (14). Both Ser-32 and Ser-36 are positioned within potential phosphorylation sites for CKII; however, it has not been possible to demonstrate a role for CKII phosphorylation in the N-terminal, signal response domain (46a). The fact that mutation of C-terminal CKII phosphorylation sites completely blocked constitutive phosphorylation of IκBα in vivo suggests that these two potential amino-terminal sites are not phosphorylated by CKII in vivo. The amino terminus of IκBα is necessary but may not be sufficient for signal-induced degradation of protein, since inducible degradation of IκBα also required the C-terminal PEST domain of the protein (11, 57, 69). In this regard, the C-terminal domain may contact the N-terminal region of the protein in vivo and act as a hinge or lever to mask the signal response domain. Further studies will delineate the roles of inducible and constitutive phosphorylation in NF-κB/IκB activation. A recent article, published during the review of this paper, also identified CKII as the kinase activity responsible for constitutive phosphorylation of IκBα in the C-terminal domain (3).

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