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# Casein Kinase II Phosphorylates IκBα at S-283, S-289, S-293, and T-291 and Is Required for Its Degradation

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**The phosphoprotein IκBα exists in the cytoplasm of resting cells bound to the ubiquitous transcription factor NF-κB (p50-p65). In response to specific cellular stimulation, IκBα is further phosphorylated and subsequently degraded, allowing NF-κB to translocate to the nucleus and transactivate target genes. To identify the kinase(s) involved in IκBα phosphorylation, we first performed an IκBα in-gel kinase assay. Two kinase activities of 35 and 42 kDa were identified in cellular extracts from Jurkat T and U937 promonocytic cell lines. Specific inhibitors and immunodepletion studies identified the IκBα kinase activities as those of the α and α′ subunits of casein kinase II (CKII). Immunoprecipitation studies demonstrated that CKII and IκBα physically associate in vivo. Moreover, phosphopeptide maps of IκBα phosphorylated in vitro by cellular extracts and in vivo in resting Jurkat T cells contained the same pattern of phosphopeptides as observed in maps of IκBα phosphorylated in vitro by purified CKII. Sequence analysis revealed that purified CKII and the kinase activity within cell extracts phosphorylated IκBα at its C terminus at S-283, S-288, S-293, and T-291. The functional role of CKII was tested in an in vitro IκBα degradation assay with extracts from uninfected and human immunodeficiency virus (HIV)-infected U937 cells. Immunodepletion of CKII from these extracts abrogated both the basal and enhanced HIV-induced degradation of IκBα. These studies provide new evidence that the protein kinase CKII physically associates with IκBα in vivo, induces multisite (serine/threonine) phosphorylation, and is required for the basal and HIV-induced degradation of IκBα in vitro.**

NF-κB is an inducible transcription factor involved in the regulation of numerous genes (5, 43). The classic NF-κB complex consists of a heterodimer of p50 (NF-κB1) and p65 (RelA) (38, 54, 68). These two subunits are members of a family of factors with homology to the c-*rel* proto-oncogene (70). In response to a variety of stimuli, combinations of these cytoplasmic c-*rel*-like factors translocate to the nucleus and transactivate specific target genes (4).

In most resting cells, NF-κB is anchored in the cytoplasm by its association with inhibitory molecules known as IκBs (43). This family of ankyrin-containing inhibitors includes IκBα (2, 3, 19, 27, 31), IκBβ (76), IκBγ (36), the p105 precursor of p50 (44, 61, 72), and the p100 precursor of p52 (66). The cloning of the cDNA of IκBα (MAD3) (31) demonstrated that aside from the IκB hallmark ankyrin repeat region, consensus sites for tyrosine phosphorylation and a phosphatidylinositol-3-kinase binding site are present. In addition, the C-terminal domain contains consensus phosphorylation sites for both protein kinase C (PKC) and casein kinase II (CKII) and a region rich in proline, glutamic acid, serine, and threonine (PEST) residues, which has been associated with rapid protein turnover (63). IκBα protein specifically inhibits the DNA binding of the p50-p65 heterodimer (22, 31) by binding to p65 and physically masking its nuclear localization signal, resulting in the cytoplasmic retention of the heterodimeric complex (8). IκBα in resting cells is a phosphoprotein with a short half-life (30 min to 3 h) (34, 48, 60, 71). Upon activation of cells with cytokines or mitogens, IκBα is further phosphorylated prior to proteasome-mediated IκBα degradation and NF-κB translocation (7, 10, 13, 18, 21, 25, 26, 33, 42, 56, 71–73), resulting in a half-life of less than 5 min (71). While it is known that the stimulus-induced phosphorylation event is necessary for rapid NF-κB activation (11, 12), the identity of the stimulus-induced kinase(s) remains elusive. Moreover, the identity and functional relevance of the kinase(s) that constitutively phosphorylates IκBα need to be determined.

Although in vitro IκBα can be phosphorylated by many different kinases, including PKC (27, 69), heme-regulated eIF2α kinase (27), protein kinase A (PKA) (27, 69), p38 (30), Raf-1 (40), a PKC-ζ-associated kinase (20), and CKII (6), the significance of this phosphorylation in vivo is unknown. Recent studies demonstrate that Ser-32 and Ser-36 are targets of inducible phosphorylation and are necessary for the subsequent degradation of this molecule in response to different stimuli (11, 12, 15, 74, 75). In addition, the carboxy-terminal region, which contains several CKII consensus motifs and the PEST sequence, is not necessary for the inducible phosphorylation of IκBα but is required for its proteolysis and subsequent NF-κB activation (12, 62, 75). Identification of both constitutive and inducible IκBα kinase activities and their role in IκBα function will enhance our understanding of NF-κB activation.

Using an in-gel kinase assay, we have identified two IκBα kinase activities of 35 to 42 kDa from Jurkat and U937 cellular extracts which correspond to the catalytic subunits (α and α′) of CKII. IκBα and CKII physically associate in vivo, and phosphopeptide mapping indicates that CKII phosphorylation of IκBα is present in vivo. Furthermore, we have determined that cell extracts and purified CKII phosphorylate IκBα at S-283, S-288, S-293, and T-291. In addition, we demonstrate that CKII is needed for the constitutive and human immunodeficiency virus (HIV)-induced degradation of IκBα in vitro.

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## MATERIALS AND METHODS

**Plasmids, recombinant IκBα, and reagents.** The IκBα MAD3 cDNA (31) plasmid was obtained from Cetus Corporation and used as a template for subsequent PCR amplification. The full-length IκBα MAD3 sequence was amplified by using wild-type (WT) primer A (CGGGATCCATGTTCCAGGCGGCCG AG), which is the 5′ sense primer, creating a *Bam*HI site upstream of the coding sequence, and WT primer B (GGAATTCCTCATA-ACGTCAGACGCTG), which is the 3′ antisense primer, creating an *Eco*RI site downstream of the coding sequence. Following digestion with *Bam*HI and *Eco*RI, this sequence was ligated into pGEX-KG (derived from pGEX-2T from Pharmacia Biotech, Piscataway, N.J.). This construct was transformed into *Escherichia coli* DH5α cells, which were grown exponentially, and after 30 to 60 min of stimulation with IPTG (isopropylthiogalactopyranoside), the full-length glutathione-*S*-transferase (GST)-IκBα (MAD3) was isolated. The purity was analyzed on a 10% acrylamide gel with sodium dodecyl sulfate (SDS) and considered to be greater than 90%.

Phorbol myristate acetate (PMA) and IPTG were obtained from Sigma (St. Louis, Mo.), and ionomycin was from CalBiochem (San Diego, Calif.). Purified sea star CKII and purified PKC (mixture of α, β, and γ) were obtained from Upstate Biotechnology Incorporated (UBI, Lake Placid, N.Y.) and stored at −70°C. Anti-human CKII (polyclonal immunoglobulin G1 [IgG1]) was also obtained from UBI and stored at −20°C. Antibodies to the catalytic subunits of CKII (α and α′) were a kind gift of Daniel R. Marshak (Cold Spring Harbor, N.Y.) (17). A polyclonal anti-IκBα (MAD3) serum was generated as previously described (48). Heparin (H-3149) was obtained from Sigma, and solutions were made at an average molecular weight of 16,000.

**Cell culture, HIV infection, and stimulation, and cell extraction.** Jurkat T cells obtained from the American Type Culture Collection were grown in RPMI 1640 (Gibco BRL, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (Intergen, Purchase, N.Y.), 100 U of penicillin/streptomycin per ml, and 2 mM L-glutamine and grown to a density of $4 \times 10^5$ to $6 \times 10^5$ cells per ml. PMA (20 ng/ml) and ionomycin (3.5 μg/ml) were used to stimulate for 5 min at room temperature. U937 promonocytic cells, obtained from the American Type Culture Collection and grown in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum, 100 U of penicillin/streptomycin per ml, and 2 mM L-glutamine, were infected with the HIV-1 LAV-Bru strain as previously described (1, 48, 57) and studied on days 20 to 90 postinfection. During this period, infected cells were 95% viable, and more than 80% of the cells expressed cytoplasmic HIV p24, as previously described (48).

To obtain total cellular protein, cells were washed with cold phosphate-buffered saline (PBS), resuspended in whole-cell extract (WCE) buffer (25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.7], 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM dithiothreitol [DTT], 20 mM β-glycerophosphate, 0.1 mM $Na_3VO_4$, 2 μg of leupeptin per ml, 100 μg of phenylmethylsulfonyl fluoride [PMSF] per ml), rotated at 4°C for 15 min, and centrifuged at 12,000 × *g* for 15 min (35). The resultant supernatant contained total cellular protein. The amount of cellular protein present in the clarified supernatant was calculated by using the Bio-Rad protein assay.

**Western immunoblotting, immunoprecipitation, and immunodepletion.** For Western blotting, equal amounts of cellular extract protein obtained as described above were loaded for each sample. These proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (10 to 12%) and transferred to Immobilon-P membranes (Millipore, Bedford, Mass.) by standard procedures. Immunoblotting was done with specific antibodies and visualized by using the ECL Western blotting detection kit (Amersham, Buckinghamshire, England).

The immunodepletion of CKII from cellular extracts was performed as previously published (17). Cellular extract (200 μg) was diluted as was done for the anchoring step of the in vitro kinase assay and subjected to three rounds of immunodepletion with antibodies to the catalytic subunits of CKII (α and α′). Each round included a 1-h incubation with the antibody at 4°C and then a 1-h incubation with protein G-agarose (Gibco BRL) at 4°C. Each cleared supernatant was used for the next round of immunodepletion. After three rounds, the cleared supernatant was diluted and subjected to a final overnight incubation with protein G-agarose. Equal amounts of each immunodepleted (or mock-immunodepleted) sample were then subjected to an in vitro kinase assay. This included the addition of 20 μM ATP, 300 ng of GST-IκBα as the substrate, and 5 μCi of [γ-$^{32}$P]ATP, followed by the assay described below.

For the immunoprecipitation experiments, 1 mg of cellular extract was incubated with polyclonal anti-IκBα (MAD3) serum or the appropriate prebleed serum for 90 min at 4°C. Protein G-agarose was then added and incubated for 1 h at 4°C. The beads were then washed, and the protein was eluted. These eluants were separated by SDS-PAGE, transferred to an Immobilon-P membrane (Millipore), and immunoblotted with anti-human CKII (UBI).

**In-gel kinase assay.** The in-gel kinase assay was performed as originally described by Kameshita and Fujisawa (37) with modifications from Hibi et al. (35). Total cellular protein (200 μg) was resolved on a 10% polyacrylamide–SDS gel which had been polymerized in the presence of 0.5 mg of GST-IκBα (MAD3) or GST per ml. Following electrophoresis, the gel was washed twice for 30 min each with 100 ml of 20% isopropanol–50 mM HEPES (pH 7.6), twice for 30 min each with buffer A (50 mM HEPES, 5 mM β-mercaptoethanol), once for 1 h with 200 ml of buffer A–6 M urea; once for 1 h with 200 ml of buffer A–0.05% Tween 20–3 M urea; once for 1 h with 200 ml of buffer A–0.05% Tween 20–1.5 M urea; once for 1 h with 200 ml of buffer A–0.05% Tween 20–0.75 M urea; and once for 1 h at 4°C with 100 ml of buffer A–0.05% Tween 20. Next, the gel was incubated in kinase buffer [40 mM HEPES, 0.10 mM EGTA (ethylene glycol tetraacetic acid), 5 mM $Mg(CH_3COO)_2$, 0.15 mM $CaCl_2$, 50 μM ATP, 5 μCi of [γ-$^{32}$P]ATP] at 30°C for 1 h. This was followed by four washes of 500 μl each with 5% trichloroacetic acid–1% sodium $PP_i$ at room temperature. The gel was then dried and autoradiographed.

**In vitro kinase assay.** The in vitro kinase assay, designed to isolate proteins which can both bind and phosphorylate IκBα, was modified from that of Hibi et al. (35). Cellular extract (500 μg), diluted to a final concentration of 20 mM HEPES, 0.15 M NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.05% Triton X-100, 0.25 mM DTT, 10 mM β-glycerophosphate, 0.05 mM $Na_3VO_4$, 1 μg of leupeptin per ml, and 50 μg of PMSF per ml, was incubated with 10 μg of GST-IκBα as an anchor molecule and swelled glutathione-agarose (Sigma) and rotated for 3 h at 4°C. This was followed by four washes with HEPES binding buffer (20 mM HEPES [pH 7.7], 50 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.05% Triton X-100). The beads were then resuspended in 15 to 30 μl of kinase buffer (20 mM HEPES [pH 7.6], 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 20 mM *p*-nitrophenyl phosphate, 0.1 mM $Na_3VO_4$, 2 mM DTT) with 20 μM ATP and 5 μCi of [γ-$^{32}$P]ATP and incubated for 20 min at 30°C. The assay was followed by two washes with HEPES binding buffer and elution with Laemmli sample buffer. For the assays with PKC as the kinase, the kinase buffer included 20 mM Tris-HCl (pH 7.5), 0.1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.31 mg of L-α-phosphatidyl-L-serine per ml, 50 ng of PMA per ml, 0.03% Triton X-100, 20 mM β-glycerophosphate, 20 mM *p*-nitrophenyl phosphate, 0.1 mM $Na_3VO_4$, 2 mM DTT with 0.1 mM ATP, and 5 μCi of [γ-$^{32}$P]ATP. For assays done without prior GST-IκBα anchoring, 50 μg of cellular extract and 3 μg of substrate were added to 15 μl of kinase buffer and incubated at 30°C for 20 to 30 min. Samples were then resolved by SDS-PAGE, dried, and visualized by autoradiography.

**In vivo phosphorylation and phosphopeptide mapping.** Cells were cultured overnight at $10^8$ cells per 50 ml of phosphate-free RPMI 1640 (Gibco) with 10% dialyzed fetal bovine serum, 100 U of penicillin/streptomycin per ml, and 2 mM L-glutamine and then labeled with 5 mCi of $^{32}P_i$ for 3 h at 37°C. Whole-cell lysates were prepared by washing once with cold PBS plus 1 mM $Na_3VO_4$, 1 mM $Na_2MoO_4$, and 1 mM PMSF, resuspending at $40 \times 10^6$ cells per ml of lysis buffer (50 mM Tris-HCl [pH 7.5], 50 mM NaCl, 2 mM EDTA, 50 mM NaF, 1% Triton X-100, 1 mM $Na_3VO_4$, 1 mM $Na_2MoO_4$, 1 mM PMSF, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, 2 μg of pepstatin A per ml, 100 mM β-glycerophosphate, 10 mg of *p*-nitrophenyl phosphate per ml), incubating on ice for 10 min, and centrifuging at 12,000 rpm for 10 min. This lysate was then incubated with rabbit polyclonal anti-IκBα (MAD3) antiserum generated against recombinant IκBα (MAD3) and immunoprecipitated with protein G-agarose (Gibco). Precipitated proteins were separated on a 12% polyacrylamide–SDS gel, dried onto 3MM paper, and autoradiographed.

IκBα or GST-IκBα bands were excised from the dried gel, rehydrated with water, and washed twice with 50% acetonitrile–50 mM $NH_4HCO_3$ at 37°C for 30 min. To each gel slice, 300 μl of 50 mM $NH_4HCO_3$ with 1 μg of V8 protease (Endoproteinase Glu-C; Promega, Madison, Wis.) per ml was added, and the gel slices were ground up and incubated overnight at 37°C. Peptides were eluted with two consecutive 30-min incubations at 37°C in 0.4 ml of 60% acetonitrile, lyophilized, washed once with water, relyophilized, dissolved in 50 μl of 95% trifluoroacetic acid, and precipitated with 950 μl of *t*-butyl-methyl ether. Precipitated peptides were washed twice with ether, air dried, then dissolved in 50 μl of pH 1.9 buffer (7.8% glacial acetic acid, 2.5% formic acid), dried, and redissolved in 10 μl of pH 1.9 buffer. Peptides were spotted on pre-coated thin-layer chromatography (TLC) cellulose plates (EM Science, Gibbstown, N.J.), electrophoresed for 30 min at 900 V in pH 1.9 buffer, and separated by TLC in phosphopeptide chromatography buffer (37.5% *n*-butanol, 25% pyridine, 7.5% glacial acetic acid) as previously described (9). The plates were then autoradiographed.

**Isolation and identification of IκBα phosphoisoforms.** Large quantities of phosphorylated GST-IκBα were obtained by incubating 1 to 2 mg of recombinant GST-IκBα with 500 U of purified CKII or 2.5 mg of U937 cellular extracts in an in vitro kinase assay. Phosphorylated samples were run on SDS-PAGE, dried onto 3MM paper, and autoradiographed. The GST-IκBα bands were excised from the dried gel, rehydrated with water, washed, and digested with cyanogen bromide (Sigma) in 70% formic acid for 2 h. The gel slices were then ground up, and the peptides were eluted from the gel with 50% formic acid–25% acetonitrile–15% isopropanol, followed by a second elution with 50% acetonitrile. A small sample of this CnBr digest was run on a 16% tricene gel along with a lane of low-molecular-weight markers. The gel was dried and autoradiographed. The remaining sample was lyophilized, and the peptides were precipitated with cold acetone. These peptides were then dissolved in 20 mM ammonium bicarbonate–20 mM NaCl (buffer A), applied to a Protein-Pak Q8HR 1000A 8-μm anion exchange column (5 by 50 mm; Waters, Milford, Mass.), and eluted by high-pressure liquid chromatography (HPLC; Shimadzu-6A) with a linear gradient to 20 mM ammonium bicarbonate–800 mM NaCl (buffer B) and a flow rate of 0.8 ml/min. The eluant was monitored at 224 nm. Fractions containing peptide were then desalted and sequenced by Edman degradation. β-Elimination and addition reactions with alkane thiols were used to identify phosphorylated amino acids (52).

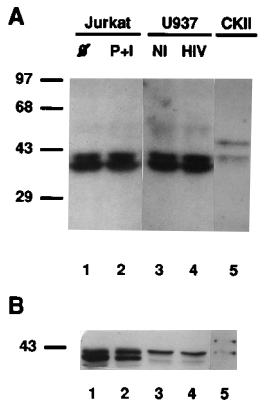


FIG. 1. Kinase(s) that phosphorylates IκBα in an in-gel kinase assay. (A) Total cellular protein (200 μg) from Jurkat and U937 cells was extracted, subjected to SDS-PAGE through a gel polymerized in the presence of GST-IκBα (0.5 mg/ml), and subsequently subjected to an in-gel kinase assay. Lanes: 5, 50 ng of CKII; Ø, unstimulated; P+I, PMA and ionomycin stimulated; NI, noninfected; HIV, HIV-1 infected. (B) Total cellular protein (40 μg per lane) was subjected to SDS-PAGE, transferred, and immunoblotted with anti-CKII antibody. Sizes are shown on the left (in kilodaltons).

**IκBα degradation assay.** The IκBα degradation assays were performed as described by Rodriguez et al. (62). Equal amounts of CKII immunodepleted or mock-immunodepleted extracts were incubated with 20 μM ATP and 150 ng of GST-IκBα at 30°C. Reactions were terminated by adding Laemmli sample buffer followed by SDS-PAGE, transfer to an Immobilon-P membrane (Millipore), and immunoblotting with polyclonal anti-IκBα (MAD3).

## RESULTS

**IκBα is phosphorylated in vitro by kinase activities of 35 to 42 kDa.** IκBα is regulated in vivo by phosphorylation (7, 18, 25, 71, 73). The sequential phosphorylation and degradation of IκBα following cellular stimulation precedes NF-κB nuclear translocation (7, 13, 18, 25, 33, 56, 72–74). To identify the kinase(s) responsible for the constitutive and/or inducible phosphorylation of IκBα, we performed an in-gel kinase assay (35, 37). Cell extracts from unstimulated and PMA-ionomycin-stimulated Jurkat T cells and from uninfected and HIV-infected U937 cells were used. Both PMA-ionomycin (13, 25) and HIV infection (48) have been shown to activate NF-κB by targeting IκBα for degradation. These cell extracts were analyzed on an SDS-PAGE gel polymerized in the presence of GST-IκBα (MAD3) or GST alone. Upon renaturation of the proteins within the gel, a kinase assay was performed. In the gel containing GST-IκBα, two radioactive protein bands with mobilities in the range of 35 to 42 kDa were detected in both Jurkat and U937 cells (Fig. 1A, lanes 1 to 4). No kinase activity was detected in the gel polymerized with GST alone (data not shown). In addition, no further kinase activity was observed by modifying the conditions of the in-gel kinase assay. These results indicate the existence of two putative IκBα kinase activities of 35 to 42 kDa which constitutively phosphorylate IκBα in these two cell lines.

A variety of kinases have been shown to phosphorylate IκBα in vitro (6, 20, 27, 30, 40, 69). Whether any of these kinases directly phosphorylate IκBα in vivo and whether they are involved in the regulation of IκBα function remain unknown. To identify the kinases present in the in-gel kinase assay, we first focused on CKII. Human CKII is a multifunctional serine/threonine protein kinase whose catalytic subunits (α and α′) are about 35 to 44 kDa in size, similar to the activities present in the in-gel kinase assay. Unlike most serine/threonine kinases, CKII phosphorylates within acidic amino acid stretches (for reviews, see references 52 and 58), and both the N- and C-terminal regions of IκBα contain several potential serine/threonine targets for CKII (31). In addition, CKII has recently been proposed as an IκBα kinase in mouse pre-B cells (6). We therefore determined whether CKII was responsible for the human IκBα kinase activities observed in the in-gel kinase assay. We first analyzed whether CKII activity could be detected in the IκBα in-gel kinase assay. Purified sea star CKII was run in an in-gel kinase assay in parallel with cellular extracts (Fig. 1A, lane 5). Two radioactive bands were detected at 39 and 44 kDa, corresponding to the two catalytic subunits (α and α′, respectively) of CKII, indicating that the activity of purified CKII can be renatured and detected in an IκBα in-gel kinase assay. The CKII activity was not detected in the gel polymerized with GST alone (data not shown). Despite the evolutionary conservation of CKII primary structure in eukaryotes, differences in the size of this protein between species have been described (15, 51, 65). To determine whether this species difference may account for the band size differences in the in-gel kinase assay, a Western blot was performed on cellular extracts from the human Jurkat and U937 cells and purified sea star CKII with an anti-CKII antibody. As shown in Fig. 1B, purified sea star CKII α and α′ forms migrated at 39 to 44 kDa, while Jurkat and U937 CKII isoforms migrated at 35 to 42 kDa. This difference in size between purified sea star CKII and the putative human cellular CKII parallels the size differences in kinase activity detected in the in-gel kinase assay, suggesting that CKII from the Jurkat and U937 extracts could be responsible for the IκBα phosphorylation.

Next, we used an in vitro kinase assay designed to isolate proteins which can both bind and phosphorylate IκBα (35). Cellular extracts from U937 cells were incubated with GST-IκBα fusion protein, allowing IκBα-binding proteins to associate. After unassociated factors were washed away, an in vitro kinase assay was performed, with the GST-IκBα now acting as the kinase substrate. This approach demonstrated an IκBα kinase activity present in the cell extracts (Fig. 2A, lane 1). GST did not associate with any IκBα kinase activity present in cell extracts, and the kinase activity associated with GST-IκBα did not phosphorylate GST (data not shown). A low concentration of heparin (50 μM), previously shown to specifically inhibit CKII activity (32), caused an almost complete block in GST-IκBα phosphorylation by the kinase(s) present in the cell extracts (Fig. 2A, lane 4). Moreover, addition of the peptide EEEAEEERRR, previously shown to inhibit CKII activity in vitro and in vivo (41), to an in vitro kinase assay with cellular extracts and GST-IκBα inhibited IκBα phosphorylation (data not shown). The specificity of heparin as a CKII inhibitor was demonstrated by its ability to block purified CKII- but not PKC-induced phosphorylation of IκBα (Fig. 2A, compare lanes 2 and 5 and 3 and 6, and data not shown). Although purified PKC phosphorylated IκBα, at least a 24-h exposure of the autoradiogram was required to visually detect it, compared with a 2-min exposure required to detect CKII- or cell extract-mediated phosphorylation of IκBα (Fig. 2A). These results point to CKII as an IκBα kinase present in the cell extracts and confirm that purified CKII can directly phosphorylate IκBα in vitro. To formally prove that CKII is responsible for the IκBα kinase activity in the human cell extracts, cell lysates were incubated with antibodies specific for the catalytic subunits of CKII (α and α′). Anti-CKII antibodies were first shown to precipitate the two catalytic domains of CKII (Fig. 2B, lane 2). Cell extracts immunodepleted with protein G-agarose beads and anti-CKII antibodies but not protein G-agarose beads alone were shown to be void of detectable IκBα kinase activity (Fig. 2C, compare lanes 3 and 4). Altogether, these results

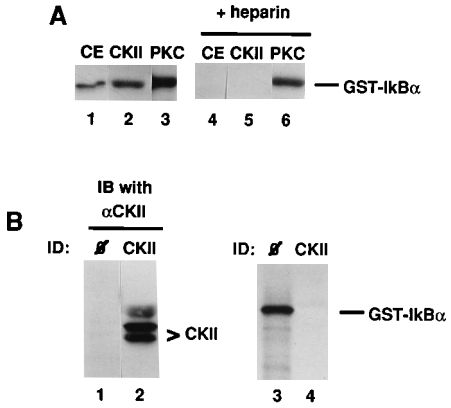


FIG. 2. Cellular CKII phosphorylates IκBα. (A) Lanes 1 and 4, U937 total cellular protein (500 μg/sample) was incubated with 10 μg of GST-IκBα. After unbound factors were washed away, an in vitro kinase assay was performed in the absence (lanes 1 to 3) or presence (lanes 4 to 6) of 50 nM heparin. Lanes 2 and 5, assays containing 30 ng of purified CKII; lanes 3 and 6, assays containing 30 ng of purified PKC (with GST-IκBα as the substrate). (B) U937 total cellular lysate (200 μg) was subjected to three rounds of immunodepletion (ID) with no antibody (Ø) or antibody to the catalytic subunits of CKII (α and α′). Immunoprecipitation with preimmune serum provided results similar to those with the no-antibody treatment. Lanes 1 and 2, immunoprecipitated proteins from the first round of immunodepletion were eluted, run on SDS-PAGE, transferred, and immunoblotted (IB) with anti-CKII antibody; lanes 3 and 4, cleared supernatants were then diluted and used in an in vitro kinase assay with 300 ng of GST-IκBα as the substrate.

confirm that CKII is a kinase responsible for phosphorylation of IκBα in human T and promonocytic cell lines.

**CKII binds to and phosphorylates IκBα in vivo.** Having demonstrated that CKII can bind IκBα in vitro (Fig. 2A, lane 1), we next tested whether CKII and IκBα interact in vivo. IκBα was immunoprecipitated from U937 cells with specific anti-IκBα antibodies and stringent conditions, analyzed by SDS-PAGE, and immunoblotted with anti-CKII antibodies. As shown in Fig. 3, anti-IκBα antibodies precipitated at least one of the catalytic subunits of CKII. Similarly, antibodies to either the α subunits (α and α′) or β subunit of CKII immunoprecipitate an IκBα-containing complex (data not shown). Studies are under way to determine whether CKII binds IκBα directly or indirectly through the NF-κB (p50-p65) heterodimer.

To determine whether the CKII interaction with IκBα in vivo results in its phosphorylation, we made phosphopeptide maps of IκBα in vivo and in vitro. IκBα proteolysis by V8 (Endoproteinase Glu-C) results in multiple peptides, allowing us to fingerprint whether the phosphorylation of IκBα by CKII in vitro matches the phosphopeptide pattern of IκBα immunoprecipitated from $^{32}$P-labeled cells. First, we determined the phosphopeptide map of recombinant IκBα (rIκBα) phosphor-

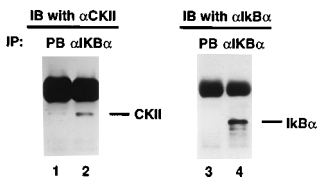


FIG. 3. IκBα binds to CKII in vivo. U937 total cellular protein (1 mg) was incubated with preimmune (PB) or polyclonal anti-IκBα (αIκBα) serum. The immunoprecipitated (IP) proteins were run on SDS-PAGE, transferred, and immunoblotted (IB) with anti-CKII antibody (lanes 1 and 2) or with an anti-IκBα antibody (lanes 3 and 4). Each molecule is marked. The exposure of lanes 3 and 4 is shorter than that for lanes 1 and 2. This experiment is representative of two additional ones.

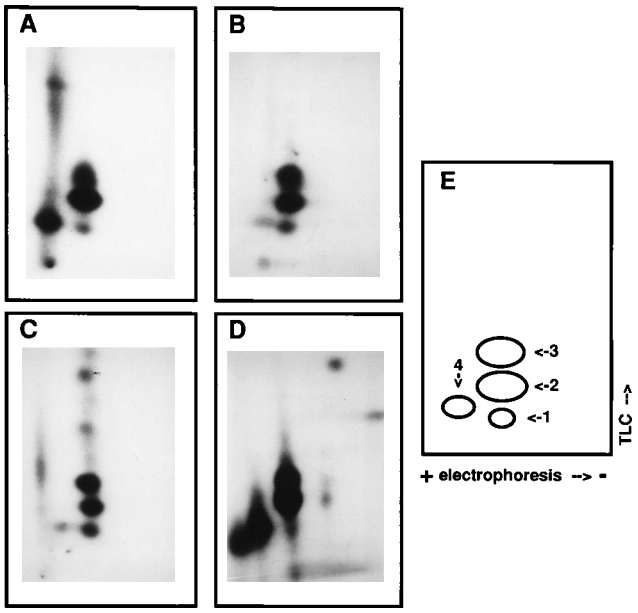


FIG. 4. Phosphopeptide analysis of IκBα phosphorylated in vitro with purified CKII or cellular lysates and of IκBα phosphorylated in vivo. GST-IκBα phosphorylated by purified CKII (A), by total cellular extracts from Jurkat T cells (B), or by lysates from Jurkat T cells which bind IκBα (C) (as explained in the legend to Fig. 2A) was subjected to digestion with V8 protease followed by electrophoresis and TLC. (D) IκBα was immunoprecipitated from $^{32}$P-labeled Jurkat T cells and subjected to digestion with V8 protease followed by electrophoresis and TLC. (E) Phosphopeptide pattern of CKII-phosphorylated IκBα. The electrophoresis and TLC directions are marked. Phosphorylated peptides are numbered 1 to 4.

ylated by CKII in vitro by using V8 protease and two-dimensional analysis. As shown in Fig. 4A, a specific phosphorylation pattern was observed that included four separate phosphorylated peptides (numbered 1 to 4 in Fig. 4E). We next determined the phosphopeptide maps of GST-IκBα phosphorylated in vitro by total cell extracts incubated or not with GST-IκBα as an anchor (Fig. 4C and 4B, respectively). In either case, each map contained the same four phosphopeptides observed in the CKII-phosphorylated IκBα map, indicating that the IκBα kinase activity within the cell extract (which also binds IκBα) is comparable to that of CKII. The same CKII-compatible fingerprint of in vitro-phosphorylated IκBα was observed with cell extracts from PMA-ionomycin-stimulated Jurkat cells and uninfected and HIV-1-infected U937 cells (data not shown). To investigate whether this CKII-specific fingerprint of IκBα can be observed in vivo, unstimulated cells were labeled with $^{32}$P and lysed, and IκBα was immunoprecipitated and analyzed on an SDS-PAGE gel. The $^{32}$P-labeled IκBα band was excised, digested with V8 protease, and subjected to two-dimensional mapping as described above, yielding the four CKII-specific phosphopeptides in addition to other minor ones (Fig. 4D). These results suggest that CKII binds to and phosphorylates IκBα both in vitro and in vivo.

**CKII phosphorylates IκBα at S-283, S-288, S-293, and T-291.** To identify the specific amino acids which are the targets of CKII, 1 to 2 mg of GST-IκBα was incubated in an in vitro kinase assay with cellular extracts from U937 cells or purified CKII. The $^{32}$P-labeled GST-IκBα was subjected to proteolysis with cyanogen bromide, which cleaves IκBα into seven distinct fragments. A small sample of CnBr-cleaved $^{32}$P-labeled GST-IκBα was then analyzed on a 16% tricene gel to determine the molecular weights of the $^{32}$P-labeled peptides

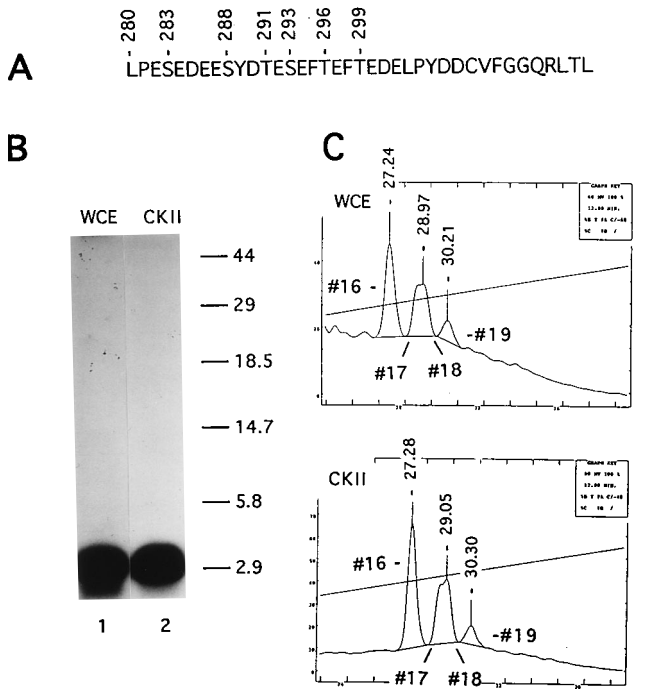


FIG. 5. Isolation and identification of CKII-phosphorylated IκBα phosphoisoforms. (A) Sequence of the IκBα C-terminal fragment which results from cyanogen bromide cleavage and contains all of the potential CKII targets in this region. (B and C) U937 total cellular extract (2.5 mg) (WCE) or purified CKII (500 U) was used to phosphorylate 2 mg of recombinant GST-IκBα. Following SDS-PAGE analysis, the phosphorylated protein was isolated and subjected to in situ cyanogen bromide cleavage. (B) A sample was then applied to a 16% tricene gel. The gel was run, dried down, and exposed to film. Molecular size markers are noted on the right side of the figure (in kilodaltons). (C) The remaining peptides were precipitated, applied to an anion exchange column, and eluted by HPLC. The numbers above the peaks are the elution times (in minutes), with fractions 16 to 19 used for further analysis. The vertical axes of the graphs represent absorbance in milliunits. The ranges are 0 to 60 mU in the upper panel and 0 to 90 mU in the lower panel. The horizontal axes represent time in minutes. In both panels, the total time represented is 12 min. The inset in the upper right corner of each panel gives the computer software (Axxiom Chromatography, Moorpark, Calif.) parameters. The parameters for the upper panel were as follows: 60 mU, 100%, 12 min, and yellow gradient. The parameters for the lower panel were as follows: 90 mU, 100%, 12 min, and yellow gradient.

phosphorylated by CKII. As shown in Fig. 5B, only a peptide of 3 to 4 kDa was identified. This would be predicted to correspond to the 37-amino-acid carboxy-terminal region of IκBα (Fig. 5A). The remainder of the $^{32}$P-labeled CnBr-cleaved sample was applied to an HPLC anion-exchange column to separate and purify the phosphopeptides for subsequent sequencing and phosphoamino acid analysis. Four different peaks corresponding to fractions 16 to 19 were observed, with only three fractions (17 to 19) containing $^{32}$P-labeled peptide (Fig. 4C). No significant differences were observed between the HPLC fractions of the IκBα peptide phosphorylated by either WCE or CKII, providing further evidence that CKII within the U937 cell extracts phosphorylates IκBα in this C-terminal region.

These phosphoisoforms (fractions 17, 18, and 19) were then sequenced and subjected to derivatization to determine specific phosphoamino acid content. All fractions contained the expected amino acid sequence for the C-terminal CnBr fragment (amino acids 280 to 314). Fraction 16 contained the CnBr fragment (amino acids 280 to 314) with none of the residues phosphorylated. Table 1 shows a summary of the data collected, comparing the amount of phosphorylated amino acid

TABLE 1. Sequences of phosphoamino acids[a]

| Residue | Ratio, phosphorylated/nonphosphorylated[b] | | | | | |
|---|---|---|---|---|---|---|
| | WCE | | | CKII | | |
| | 17 | 18 | 19 | 17 | 18 | 19 |
| Serine 283 | ++ | ++ | +++ | ++ | + | +++ |
| Serine 288 | + | + | + | + | + | + |
| Threonine 291 | − | + | ++ | − | + | ++ |
| Serine 293 | + | ++ | + | + | + | + |
| Threonine 296 | − | − | − | − | − | − |
| Threonine 299 | − | − | − | − | − | − |

[a] Peptide-containing fractions were sequenced, and phosphorylated amino acids were identified. Fraction 16 includes peptides with the sequence shown in Fig. 5A and no detectable phosphorylation. Fractions 17 to 19 contain peptides with the same sequence but with detectable phosphorylation. Symbols indicate the level of phosphorylated amino acids for every nonphosphorylated amino acid at that position. The data are summarized from two separate experiments.

[b] Ratios: −, 0.0 to 0.2; +, 0.2 to 1.0; ++, 1.0 to 3.0; +++, >3.0.

with unphosphorylated amino acid at each serine and threonine residue in fractions 17, 18, and 19. These ratios were comparable in both CKII- and cellular extract-phosphorylated samples. Fraction 17 contained a high level of phosphorylation at S-283 and lower levels at S-288 and S-293. Fraction 18 included significant phosphorylation at the same three serines, with the addition of threonine phosphorylation at amino acid 291. The most highly phosphorylated isoform was present in fraction 19, with very high levels of phosphorylation at S-283 and T-291 and moderate levels at S-288 and S-293 comparable with fractions 17 and 18. This indicates that both purified CKII and CKII present in cellular extracts phosphorylate IκBα at S-283, S-288, S-293, and T-291, with undetectable phosphorylation at T-296 and T-299. These data provide additional evidence that the serine/threonine kinase CKII is responsible for multisite phosphorylation of IκBα at its C terminus.

**CKII is required for the constitutive and HIV-induced degradation of IκBα in vitro.** While the data presented so far establish for the first time that specific amino acids within IκBα are targets of an identified IκBα kinase, it still remains to be determined whether this kinase plays any role in IκBα function. We have shown that CKII constitutively phosphorylates IκBα at specific serine and threonine residues in the C-terminal region of the IκBα molecule, which is rich in proline (P), glutamic acid (E), serine (S), and threonine (T) residues. This PEST motif is a hallmark of proteins which have short half-lives (63). More importantly, previous studies have indicated that the C terminus of IκBα containing the PEST sequence is required for the degradation of this molecule in both in vitro and in vivo models (12, 62, 75). From this, we hypothesized that the constitutive phosphorylation of IκBα by CKII may be necessary for the turnover of the IκBα molecule. To test this hypothesis, an in vitro IκBα degradation assay was used (62). In this in vitro system, the kinetics and characteristics of IκBα degradation parallel those described in vivo (62). To test whether CKII plays a role in the basal level of IκBα degradation in vitro, cell extracts which had been mock immunodepleted or CKII immunodepleted were incubated with GST-IκBα and ATP. At different time points, the reactions were terminated, analyzed by SDS-PAGE, and immunoblotted for IκBα. As shown in Fig. 6A, mock-immunodepleted extracts from U937 cells showed a time-dependent degradation, which is similar to that observed for IκBα in vivo (48). However, CKII-immunodepleted extracts did not induce any detectable degradation within the same time points (Fig. 6B). Degradation of GST-IκBα could also be precluded when the CKII

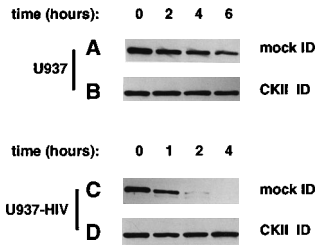


FIG. 6. Immunodepletion of CKII prevents IκBα degradation in unstimulated and HIV-infected U937 cells. Unstimulated U937 (A and B) or HIV-infected U937 (C and D) samples were generated as described in the legend to Fig. 2B. Equal amounts of CKII-immunodepleted (ID) (B and D) or mock-immunodepleted (A and C) extracts were incubated with 150 ng of GST-IκBα. At the given times, the reactions were terminated by boiling in sample buffer. Samples were then analyzed on SDS-PAGE, transferred to an Immobilon membrane, and immunoblotted with polyclonal anti-IκBα antibodies.

inhibitor heparin was added to mock-depleted samples (data not shown). This suggests that CKII participates in the turnover of IκBα.

We have previously shown that HIV infection of U937 cells results in an increased turnover of IκBα (48), providing a mechanism by which NF-κB activity is persistently upregulated during HIV infection. To test whether CKII participates in this virus-induced increase in IκBα degradation, cell extracts from HIV-infected U937 cells were depleted or not of CKII in parallel to the above experiment. As expected, mock-depleted HIV extracts were able to degrade GST-IκBα with faster degradation kinetics than observed with extracts from uninfected cells (compare Fig. 6A and C). More importantly, extracts from HIV-infected U937 cells depleted of CKII were unable to degrade GST-IκBα (Fig. 6D). Together, these data suggest that CKII is required for the constitutive degradation of IκBα as well as being a prerequisite for the HIV-induced degradation of IκBα.

## DISCUSSION

A variety of kinases have been shown to phosphorylate IκBα in vitro (6, 20, 27, 30, 40, 69). However, their role in the in vivo phosphorylation of IκBα and, more importantly, in the regulation of IκBα function has remained elusive. Data presented here indicate that CKII should be classified as an IκBα kinase, in that CKII can phosphorylate IκBα in vitro, it is associated with IκBα in vivo, and the phosphopeptide map of IκBα immunoprecipitated from human cell lines contains the same phosphopeptides as the map of IκBα phosphorylated by CKII in vitro. In addition, we provide evidence to establish an association between CKII-mediated phosphorylation and the degradation of IκBα.

Our studies, while confirming those of Barroga et al. (6) that indicated that CKII phosphorylates IκBα at its C terminus in murine pre-B cells, provide new information as to the specific amino acids that are phosphorylated by CKII in addition to the role of this kinase in the degradation of IκBα. S-283 appears to be the amino acid with the highest level of phosphorylation, while phosphorylation at S-288 and S-293 is consistent, albeit lower, in each phosphoisoform. T-291 phosphorylation is evident in two of the three phosphoisoforms isolated, one at relatively high levels, while T-296 and T-299 do not appear to be targets of CKII. Within each of the multiple other CKII substrates identified to date (see below), one or at most two amino acids are the target of CKII. However, our data indicate that CKII is responsible for multisite phosphorylation within a region which is already very negatively charged. Future studies will need to address the number of amino acids phosphorylated in vivo and whether a specific sequential phosphorylation is required within the identified target amino acids. The fact that, within what appeared to be a single phosphopeptide in the tricene gel analysis, multiple C-terminal phosphoisoforms could be detected by anion exchange HPLC is consistent with the in vitro and in vivo phosphopeptide maps. Such maps indicate that the same phosphopeptide has different degrees of phosphorylation and suggest that the same amino acids are phosphorylated in vivo and in vitro by CKII. Data from both phosphopeptide maps and HPLC analysis suggest that while there are four potential target amino acids of CKII, different combinations of amino acids may be phosphorylated in any given phosphoisoform. For example, within each phosphoisoform peak (Fig. 5C), S-288 and S-293 each contained approximately one phosphorylated serine for each unphosphorylated serine, suggesting that each of these isoforms may contain a phosphorylated residue at either S-288 or S-293. The inability to further separate peaks 17 and 18 precluded an exact identification of each phosphoisoform; however, it would suggest that they are extremely similar and perhaps indicate that each form contains that same number of phosphorylated amino acids but at different residues. Fraction 19 appears to represent the most highly phosphorylated peptide, with phosphorylation at S-283, T-291, and S-288 and/or S-293. Preliminary evidence indicates that the phosphopeptide present in fraction 19 is the most susceptible to degradation. In addition to the phosphopeptide maps, sequencing of the amino acids phosphorylated in vitro by CKII and cell lysates has provided the formal proof to support the idea that the IκBα kinase activity present in cell extracts is CKII. Point mutations within the CKII phosphorylation sites are being generated to further elucidate the potential hierarchy of phosphorylation events and their exact role in IκBα phosphorylation by CKII in vivo.

In spite of the ability of CKII to phosphorylate several relevant cellular proteins, including Jun (41), Myc (46), Myb (45), c-Erb-A (28), adenovirus E1A (14), human papillomavirus type 16 E7 (24), CRE (39), p53 (49), SRF (47), and simian virus 40 large T antigen (29), the function of CKII in cell biology remains elusive. Its most clearly defined role to date is that of a negative regulator of c-Jun. In nonstimulated fibroblasts and epithelial cells, CKII-specific phosphorylation of sites proximal to the c-Jun DNA-binding site prevents AP-1 activation (41). In these cells, it is the constitutive CKII activity which is functionally significant in causing the attenuation of AP-1 activity. The identification of CKII as an IκBα kinase prompted us to investigate whether it plays a role in the regulation of IκBα. The amino acids of IκBα shown in this study to be phosphorylated by CKII are located within a sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T), a PEST motif. Multiple proteins which have short half-lives (less than 2 h) have been shown to contain PEST sequences and to be phosphorylated by CKII (53, 63). Although controversial, it has been proposed that CKII phosphorylates residues within the PEST region of proteins, inducing the proteolysis of the polypeptide substrate (63).

Knowing that the basal level of IκBα degradation in unstimulated cells is relatively high (less than 3 h) and that this degradation is a prerequisite for normal cellular NF-κB activation, we investigated the potential role of CKII in this degradation process. For this, we used an in vitro assay shown by other groups to closely simulate the in vivo degradation process of IκBα (62). In this system, rIκBα incubated with cellular extracts undergoes proteolysis with kinetics comparable to those of native IκBα in vivo. This degradation could be prevented by immunodepleting CKII from the extracts or inhib-

iting CKII activity with the specific inhibitor heparin. This indicates that CKII is a necessary element for the usual basal turnover of IκBα and provides the first indication of a functional role for this IκBα kinase. In addition, we have previously reported an increased level of IκBα degradation in HIV-infected U937 cells (48), compared with that in uninfected cells, which results in the persistent activation of NF-κB in infected cells (1, 57, 64). In the in vitro degradation system, IκBα proteolysis induced by cell extracts from HIV-infected U937 cells was faster than in uninfected cells, and again, depletion of CKII from the cell extracts from HIV-infected U937 cells resulted in complete inhibition of IκBα degradation. This suggests that CKII is also necessary for IκBα degradation in HIV-infected cells. At this point, we are unable to determine whether this increase in degradation of IκBα in HIV-infected cells is due to the upregulation of a CKII-specific mechanism or if CKII is a prerequisite for a separate HIV-induced mechanism of IκBα degradation. Future studies will need to address the function of IκBα molecules containing mutations of S-283, S-289, S-293, and T-291 in vivo and to determine the exact role of this kinase in the constitutive and inducible degradation of IκBα.

In conclusion, this work supports CKII as an IκBα kinase and has identified its target residues. More importantly, it ascribes a functional role for CKII in the degradation of IκBα and, presumably, NF-κB activation. Linking CKII to NF-κB regulation through its interaction with IκBα may in part explain the oncogenic effects of overexpressed CKII (67) and, additionally, its crucial function in cell survival, exemplified by the fact that disruption of the catalytic subunits of CKII in *S. cerevisiae* results in lethality (55).

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