

Constitutive Phosphorylation of I κ B α by Casein Kinase II Occurs Preferentially at Serine 293: Requirement for Degradation of Free I κ B α

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I κ B α is a phosphoprotein that sequesters the NF- κ B/Rel transcription factors in the cytoplasm by physical association. Following induction by a wide variety of agents, I κ B α is further phosphorylated and degraded, allowing NF- κ B/Rel proteins to translocate to the nucleus and induce transcription. We have previously reported that the constitutive phosphorylation site resides in the C-terminal PEST region of I κ B α and is phosphorylated by casein kinase II (CKII). Here we show that serine 293 is the preferred CKII phosphorylation site. Additionally, we show compensatory phosphorylation by CKII at neighboring serine and threonine residues. Thus, only when all five of the serine and threonine residues in the C-terminal region of I κ B α are converted to alanine (MutF), is constitutive phosphorylation abolished. Finally, we show that constitutive phosphorylation is required for efficient degradation of free I κ B α , in that unassociated MutF has a half-life two times longer than wild-type I κ B α . A serine residue alone at position 293, as well as aspartic acid at this position, can revert the MutF phenotype. Therefore, the constitutive CKII phosphorylation site is an integral part of the PEST region of I κ B α , and this phosphorylation is required for rapid proteolysis of the unassociated protein.

The NF- κ B/Rel family of transcription factors are key regulators of a variety of genes involved in the immune and inflammatory responses, growth, differentiation, and development (5, 28, 33, 36). NF- κ B/Rel proteins are sequestered in an inactive form in the cytoplasm through their association with an inhibitory protein called I κ B (3, 4, 6, 8). Stimulation of the cells with a variety of agents results in a rapid degradation of I κ B, allowing the translocation of NF- κ B/Rel proteins to the nucleus where they can activate the transcription of target genes containing the decameric κ B-DNA binding site (7, 12, 15, 16, 20, 31, 34). This unique and rapid mechanism of activation makes members of the NF- κ B/Rel family of transcription factors potent and pleiotropic gene activators.

The diversity of agents that can activate the NF- κ B complex includes cytokines like tumor necrosis factor alpha (TNF- α), interleukin-1, phorbol esters, bacterial lipopolysaccharides (LPS), DNA damaging agents, double-stranded RNA, hydrogen peroxide, and chemicals that generate reactive oxygen intermediates (2, 5). Although many of these activators utilize different signal transduction pathways, they all converge on one common target, the NF- κ B/I κ B α complex, resulting in the loss of I κ B α proteins. Two domains of I κ B α have been shown to be involved in signal dependent proteolysis, the N terminus, which is phosphorylated at Ser-32 and/or Ser-36 in a signal-dependent manner (11), and the C terminus, which contains the PEST region (11, 32).

While it has long been known that I κ B α and its homolog in chicken, pp40, are found in resting cells as phosphoproteins (17, 21), little is known about the sites of phosphorylation or the kinase involved and its function. Recently, we and others have demonstrated that the constitutive phosphorylation site of I κ B α resides in the C terminus (9, 32). We have identified

casein kinase II (CKII) as the constitutive I κ B α kinase by purification and immunodepletion experiments (9). Here we show that while CKII is capable of phosphorylating several serines and threonines in the C terminus, serine 293 is the preferred phosphorylation site. The data also show that the constitutive phosphorylation site is an integral part of the PEST sequences and that such phosphorylation is required for rapid degradation of free I κ B α .

MATERIALS AND METHODS

I κ B α mutants and glutathione S-transferase (GST) fusion proteins. Mutants were generated by PCR and confirmed by sequencing. The pT7GT fusion protein expression plasmids were transformed into the *Escherichia coli* strain BL21(DE3) (Novagen). Bacterial cultures (1 liter) were grown to an optical density at 600 nanometers of 1.0 and then induced for 3 h with 0.5 mM isopropyl- β -D-thiogalactoside (IPTG). Cells were resuspended in 30 ml of phosphate-buffered saline (PBS) containing 0.1% Triton X-100, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 10 μ g of aprotinin per ml, and 20 μ g of leupeptin (Sigma) per ml and sonicated on ice. The soluble fractions were incubated with 1.0 ml of glutathione-Sepharose beads (1:1) (Pharmacia) for 1 h at 4°C, after which they were washed four times with 50 ml of the above lysis buffer.

Preparation of cytoplasmic extracts. The mouse pre-B cell line, 70Z/3, stably harboring the CD14/LPS receptor, 70Z/3-hCD14 (19), was kindly provided by R. J. Ulevitch (Scripps Research Institute). The cells were grown in RPMI (GIBCO) medium containing 10% fetal calf serum (FCS), 10 μ g of penicillin per ml, 100 μ g of streptomycin per ml, 800 μ g of G418 per ml, and 50 μ M β -mercaptoethanol. The cells were grown to a density of 10⁶/ml, in some cases by stimulation with 10 ng of LPS (Sigma) per ml for 30 min, and were harvested by resuspending in PBS buffer (10 mM Na₂HPO₄, 150 mM NaCl, 2.7 mM KCl) containing 0.1% Triton X-100, 1 mM DTT, 10 μ g of leupeptin per ml, 10 μ g of aprotinin per ml, 0.1 mM phenylmethylsulfonyl fluoride, and 0.2 mM Na₃VO₄. Whole cell extracts were cleared by centrifugation at 10,000 \times g for 10 min.

In vitro kinase assays. Cell extracts (100 μ g) or purified CKII (20 ng) P18 fraction (9) or commercially available CKII (20 ng) (Calbiochem) were diluted to 1.0 ml in 1 \times PBS, 0.1% Triton X-100, 0.1% Tween 20, 1 mM DTT. To this we added 10 μ g of GST or GST fusion proteins bound to glutathione-Sepharose beads. The mixture was rotated at 4°C for 1 h, after which the beads were pelleted and washed three times with 1 ml of the 0.1% Triton X-100 PBS binding buffer and then once with 1 ml of the HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) kinase buffer without ATP (20 mM HEPES-KOH [pH 7.4], 10 mM MgCl₂, 10 mM MnCl₂, 1 mM DTT). After washing, the beads were

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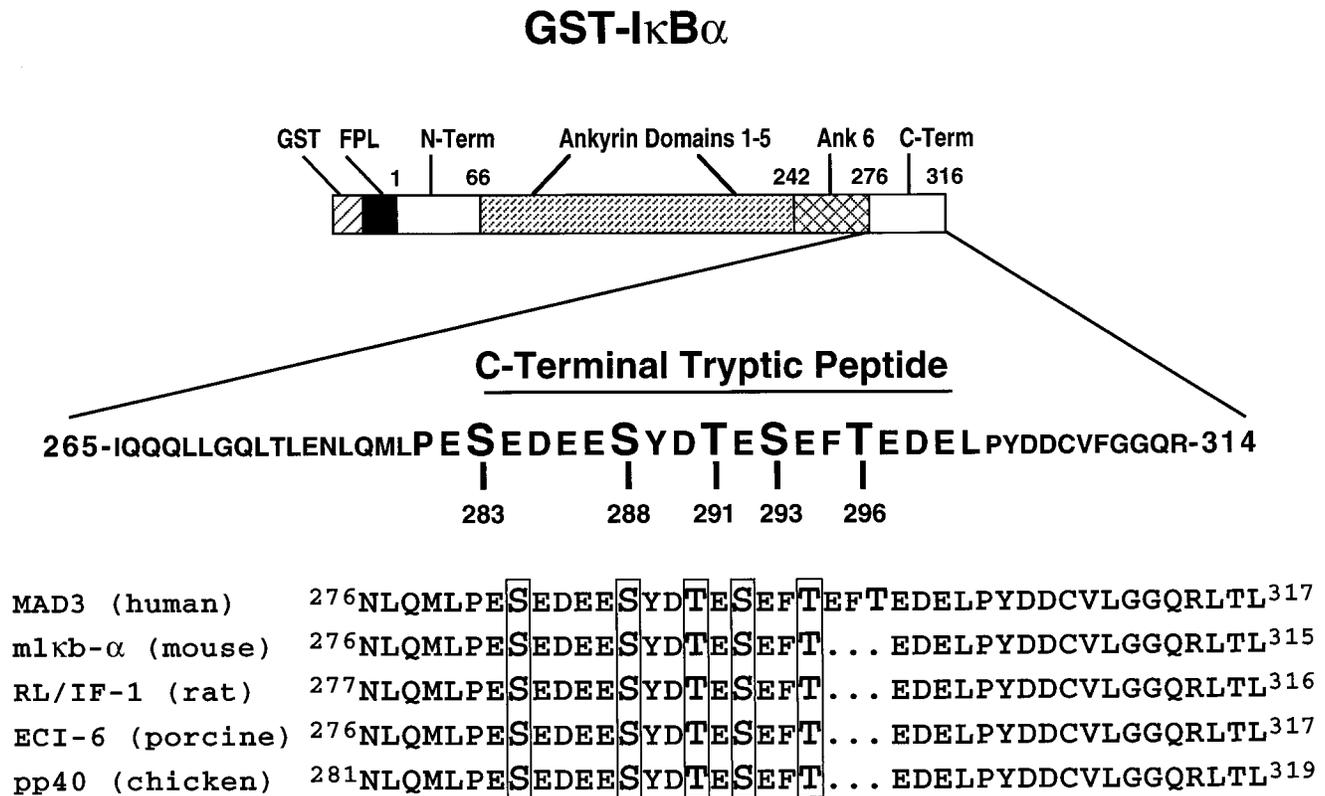


FIG. 1. Schematic representation of I κ B α and the conserved CKII phosphorylation sites. The sequences of the mouse I κ B α C-terminal tryptic peptide containing the CKII consensus sites are shown. The serine and threonine residues are in bold letters. The PEST sequences in I κ B α proteins identified in human, mouse, rat, porcine, and chicken cells are shown to indicate their conservation.

resuspended in 20 μ l of the kinase buffer containing 25 μ M ATP and 2 μ Ci of [γ -³²P]ATP. After incubation for 15 min at 37°C, the reaction was terminated by washing three times with 1 ml of the 0.1% Triton X-100 PBS binding buffer. The phosphorylated proteins were eluted from the beads with 20 μ l of 2 \times Laemmli sample buffer, resolved on sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-10% PAGE) gels, and transferred to an Immobilon-P membrane (Millipore), and this was followed by autoradiography.

In vivo ³²P labeling and immunoprecipitation. WEHI and 70Z/3-hCD14 cells (2.8×10^7) were incubated for 6 h at 37°C in 3 ml of PO₄-minus Dulbecco minimal essential medium (DMEM) containing 10% dialyzed fetal calf serum (FCS) (GIBCO BRL), 1 mM sodium pyruvate, 100 μ M nonessential amino acids, 0.29 mg of L-glutamine per ml, and 0.8 mCi of [³²P]orthophosphate. Whole cell extracts were prepared and the I κ B α protein was immunoprecipitated with a 1:200 dilution of anti-I κ B α serum (25) raised against the N-terminal 60 amino acids of I κ B. Immunoprecipitated proteins were fractionated by electrophoresis in an SDS-12.5% PAGE gel.

Two-dimensional-TLC phosphopeptide and phosphoamino acid analysis. Phosphopeptide and phosphoamino acid analysis were performed as described (10). Briefly, purified phosphoproteins were digested either with trypsin or chymotrypsin (Worthington) or hydrolyzed with 6 N HCl at 110°C and then spotted on cellulose thin-layer chromatography (TLC) plates. Phosphopeptide maps were obtained first with pH 1.9 electrophoretic buffer (horizontal) and then with pH 8.9 phosphochromatography buffer (vertical). Phosphoamino acid maps were obtained first with pH 1.9 electrophoretic buffer (horizontal) and then with pH 3.5 electrophoretic buffer (vertical) which had been sprayed with ninhydrin. Both analyses were followed by autoradiography. Quantitation of the P-Ser/P-Thr ratio was done by phosphoimager (Molecular Dynamics).

Transient transfection and I κ B α stability assay. The I κ B α and mutant cDNAs were removed from the pT7GT constructs, cloned into the pCMX expression plasmid (26), and used in transient-transfection assays. Confluent 293 cells were split 1:10 in DMEM medium with 10% FCS and transfected the following day by calcium phosphate (13). Due to its stability, a 5:1 ratio of I κ B α :MutF DNA (see below) was used to equilibrate the amount of labeled protein at the zero time point. The transfected cells (5×10^6) were washed twice in PBS and incubated in 3.5 ml of Cys- and Met-DMEM with 10% dialyzed FCS for 1 h at 37°C. Next, 0.5 mCi of [³⁵S]methionine (Dupont) per ml was added to the cells, which were then incubated for 1 h at 37°C. The cells were washed three times with DMEM and then chased in normal 293 medium for the indicated

times before being processed for immunoprecipitation and SDS-PAGE as described above. In the case of the Rel A coimmunoprecipitation experiment, the cells were lysed in radioimmunoprecipitation assay buffer without SDS and antibodies raised against p65 (27) were used to immunoprecipitate complexed I κ B α . The proteins were fractionated on an SDS-12% PAGE gel; this was followed by autoradiography. Quantitation of the I κ B α half-life was done by phosphoimager. The data presented are the averages of three independent experiments.

RESULTS

I κ B α is constitutively phosphorylated at serine 293. We have previously shown that mouse or human I κ B α is constitutively phosphorylated at its C terminus by CKII (9, 21). Figure 1 shows both a diagram of the different regions of mouse I κ B α and the conserved homology of CKII sites among I κ B α proteins from various species. There are five potential phosphorylation sites, i.e., Ser-283, Ser-288, Thr-291, Ser-293, and Thr-296. We generated mutant GST-I κ B α fusion proteins (Fig. 2A) in which each of these sites was mutated individually to alanine. Additionally, we generated an I κ B α protein where all five sites were mutated to alanine (MutF). To identify the constitutive phosphorylation site we analyzed the phosphoamino acid composition of our five point mutant GST-I κ B α proteins by in vitro kinase reaction and compared them with the wild type. Although all the fusion proteins were labeled with equal efficiency (Fig. 2B), the Ala-293 mutant had an altered phosphoamino acid composition, from a P-Ser/P-Thr ratio of about 4:1 to a ratio of about 2:1 (Fig. 2C and D). This led us to suspect that serine 293 was the preferred phosphorylation site and that in its absence, compensatory phosphorylation could occur.

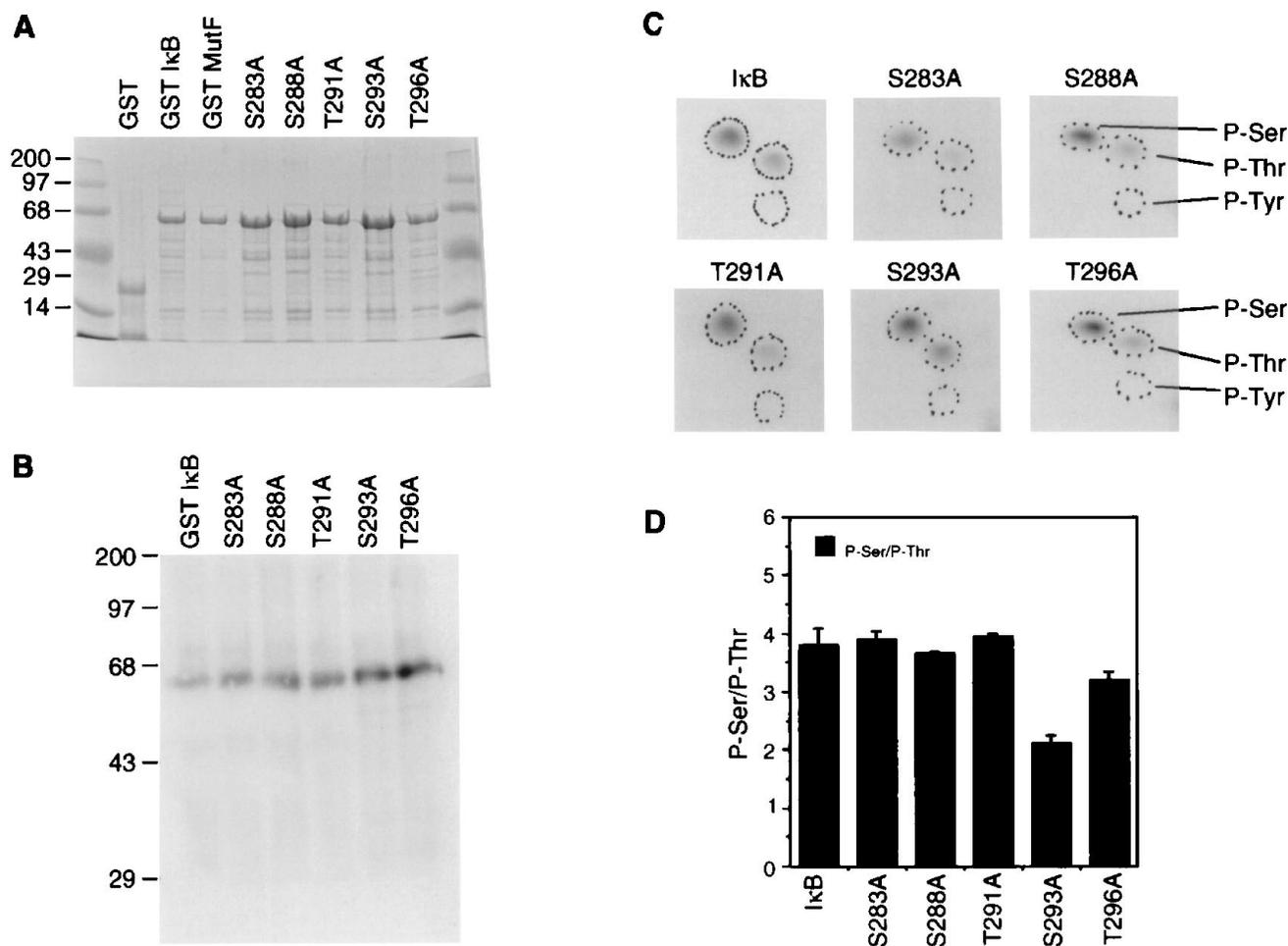


FIG. 2. Identification of the constitutive IκBα phosphorylation site. (A) Bacterially expressed GST fusion proteins were produced and their integrity was checked in a Coomassie blue stained gel. These proteins were then ^{32}P -phosphorylated in vitro with whole cell extracts from unstimulated 70Z/3-hCD14 cells. The labeled proteins were resolved on an SDS-10% PAGE gel; this was followed by autoradiography. (B) Duplicate experiments with normalized protein inputs revealed that the labeling efficiencies of all the mutants, except MutF, were equal. (C) A phosphoamino acid analysis was performed as previously described (10) and (D) the P-Ser/P-Thr composition of the labeled proteins was calculated by phosphoimagery. The data are the averages of three independent experiments.

In order to test this prediction we performed a two-dimensional tryptic peptide analysis on these proteins. We first determined the two-dimensional tryptic peptide map of in vivo ^{32}P -labeled mouse IκBα (Fig. 3A) from WEHI 231 cells. A single phosphopeptide with mobility consistent with the C-terminal CKII peptide (Fig. 3B) was identified. To confirm that this peptide (peptide C; constitutive site) originated from the C-terminal region, we compared maps from mutant IκBα proteins. When GST-IκBα protein is phosphorylated in vitro with extracts from unstimulated 70Z/3-hCD14 cells and subjected to two-dimensional tryptic analysis, peptide C can be identified (Fig. 2C). Additionally, a phosphopeptide originating from the GST fusion protein can also be identified. As expected, peptide C cannot be identified when GST-MutF is phosphorylated with unstimulated extracts, while the GST-specific phosphopeptide remains (Fig. 3D). Consistent with the results of the phosphoamino acid analysis, the two-dimensional tryptic maps of all five single-point mutant phosphoproteins appear similar to the wild type (data not shown), again implicating compensatory CKII phosphorylation. As an example GST-Ala-293 is presented (Fig. 3E). Next we show that a serine residue at position 293 is sufficient for ^{32}P labeling of the C-terminal

peptide. Using the IκBα mutant Ser-293 (Fig. 3F), in which all the phosphorylation sites except serine 293 were mutated to alanine, we can still see efficient labeling of the C-terminal peptide. Finally, since chymotrypsin digests the CKII phosphorylation sites into three different peptides, we were interested in determining the mobility of the labeled C-terminal chymotryptic peptide. As can be seen by comparing the GST-IκBα and the GST-MutF chymotryptic maps (Fig. 3G and H), the labeled peptide has the mobility of a neutral, low-molecular-weight peptide. This result eliminates serines 283 and 288 as candidates for the preferential constitutive phosphorylation site, since they reside on a chymotryptic fragment larger than 5 kDa. As expected, phosphoamino acid analysis of the labeled C-terminal chymotryptic peptide revealed that phosphorylation occurs on serine (data not shown), again arguing that Ser-293 is the phosphorylation site.

To further characterize the precise constitutive phosphorylation site, we first determined the phosphoamino acid composition of IκBα from resting WEHI cells in vivo. Virtually all the phosphate in IκBα is covalently bound to serine (Fig. 4A). Analysis of peptide C obtained from Fig. 3C (GST-IκBα plus unstimulated extracts) also revealed predominant phosphory-

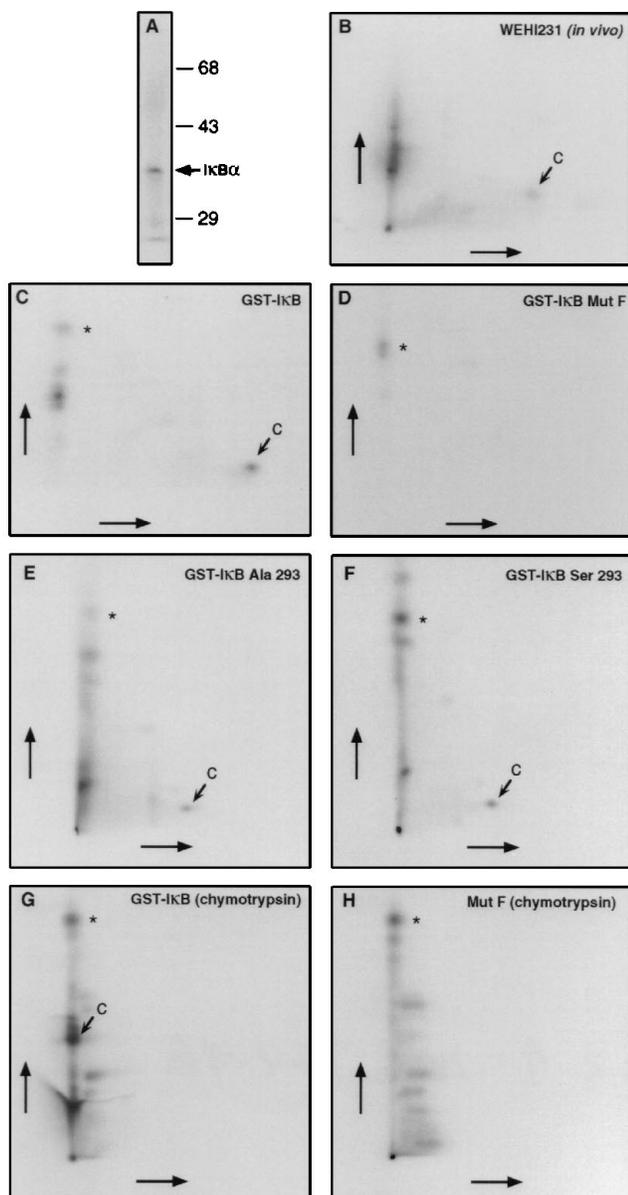


FIG. 3. Phosphopeptide mapping of I κ B α . Autoradiograms of *in vivo*-immunoprecipitated I κ B α from WEHI 231 cells metabolically labeled with [32 P]orthophosphate. (A) The I κ B α protein was separated by SDS-PAGE, transferred to a membrane, and exposed to autoradiography. (B) The band was cut out, digested with trypsin, and the peptides were mapped by two-dimensional TLC. (C to H) GST-I κ B α fusion proteins were labeled with [γ - 32 P]ATP *in vitro* by using unstimulated 70Z/3-hCD14 cell extracts. Following SDS-PAGE and digestion with trypsin (C to F), or chymotrypsin (G and H), the peptides were separated by two-dimensional TLC. The GST fusion proteins used were I κ B α (C and G), Mut F (D and H), Ala-293 (E), and Ser-293 (F). The short arrow and the "C" indicate the constitutive phosphopeptide and the asterisk indicates the GST phosphopeptide. The first dimension of the mapping was done in pH 1.9 electrophoretic buffer in the direction indicated by the horizontal arrow. The second dimension was run in pH 8.9 phosphochromatography buffer in the direction indicated by the vertical arrow.

lation at serine (Fig. 4B), presumably representing phosphorylation of Ser-293. Interestingly, peptide C from Fig. 3E (where Ser-293 is converted to Ala but the rest of the sites are intact) shows, in addition to serine, some phosphorylation of threonine residues. As expected, peptide C from Fig. 3F (Ser-293, in

which all of the other four possible sites have been mutated) shows only serine phosphorylation (Fig. 4D).

The data are shown with extracts from unstimulated cells, but similar results were obtained if purified CKII or a highly purified fraction (p18) from unstimulated cells (9, 33a) was used for phosphorylation. Thus, on the basis of phosphopeptide and amino acid mapping experiments, we conclude that (i) serine 293 is the preferred CKII phosphorylation site of I κ B α , and (ii) compensatory CKII phosphorylation in the C terminus of I κ B α occurs in the absence of serine 293.

C-terminal phosphorylation of I κ B α is required for efficient proteolysis. To determine the function of the constitutive phosphorylation of I κ B α by CKII, we developed a transient-transfection system in 293 cells whereby the stability of free I κ B α and mutant proteins could be studied. We have analyzed the constitutive phosphorylation status of the transfected I κ B α by *in vivo* labeling and determined that it closely resembles that of the endogenous protein (data not shown). To ensure that relatively equal amounts of free exogenously introduced protein were made, we metabolically labeled the transfected cells with [35 S]Met and [35 S]Cys and examined the amount of free and Rel A (p65)-associated I κ B α in the cells (Fig. 5A). In addition to testing I κ B α wild type, I κ B α -MutF, and Ser-293, we also used a MutF protein with an aspartic acid at position 293 (Asp-293). In all of the samples tested, more than 95% of the labeled I κ B α proteins could not be coimmunoprecipitated with antibodies to Rel A (p65) under native conditions. Comparison of I κ B α bands shows that the labeling efficiencies of all the sam-

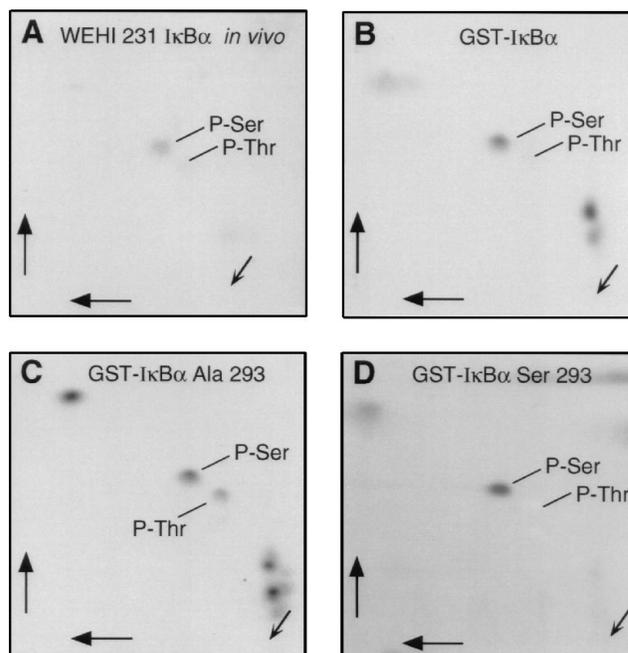


FIG. 4. Phosphoamino acid analysis of I κ B α . *In vivo* 32 P-labeled I κ B α protein from WEHI cells was isolated as previously described and hydrolyzed with 6 N HCl acid. The resulting mixture was then mapped by two-dimensional TLC (A). The locations of the phosphorylated amino acids, visualized with ninhydrin staining, are indicated. Some of the phosphopeptides seen in Fig. 3 were removed from the TLC plates, hydrolyzed, and mapped by two-dimensional TLC. The samples represent the phosphoamino acid composition of the C-terminal peptide from *in vitro* 32 P-labeled I κ B α (B), Ala-293 (C), and Ser-293 (D). The first dimension of the mapping was done in pH 1.9 electrophoretic buffer in the direction indicated by the horizontal arrow. The second dimension was run in pH 3.5 electrophoretic buffer in the direction indicated by the vertical arrow. The short arrow indicates the origin.

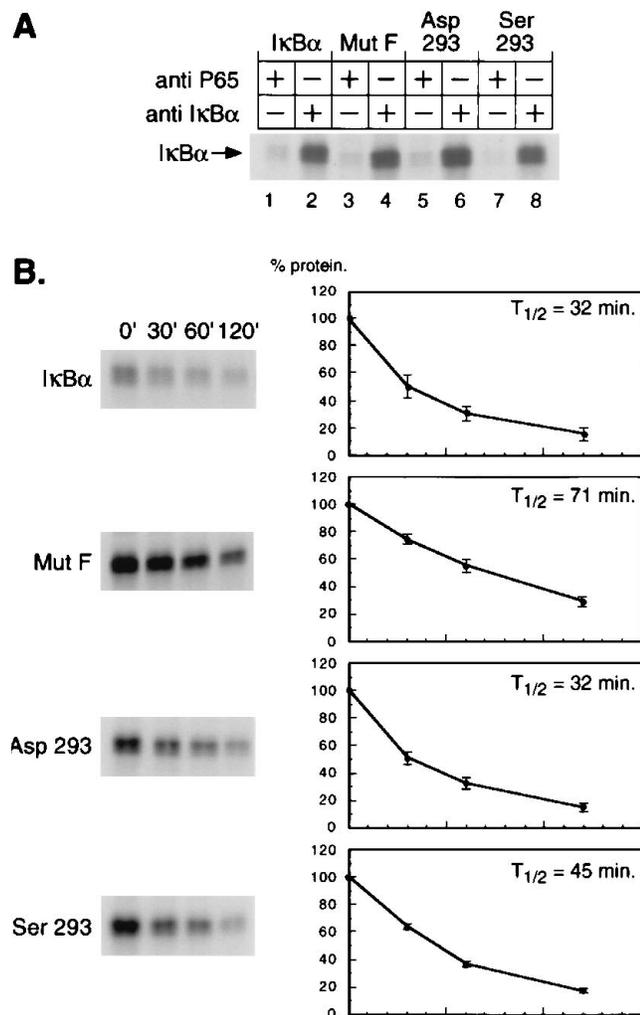


FIG. 5. Constitutive phosphorylation of free IkB α is required for efficient proteolysis in vivo. (A) 293 cells transfected with IkB α expression plasmids were metabolically labeled with [³⁵S]methionine and [³⁵S]cysteine for 1 h and lysed under native conditions. The NF- κ B-associated IkB α proteins were immunoprecipitated from the lysates with antibodies raised against p65 (lanes 1, 3, 5, and 7). The free IkB α proteins were obtained by reimmunoprecipitating the supernatants with antibodies raised against IkB α (lanes 2, 4, 6, and 8). The immunoprecipitated proteins were separated by SDS-PAGE, and the amount of bound versus free IkB α in the cells was determined by phosphoimager. For all the samples, the amount of free IkB α was greater than 95% of the total. (B) To determine the stability of the different IkB α proteins, aliquots were taken at the indicated times during the chase. Cells harvested at 0, 30, 60, and 120 min following the chase were processed for immunoprecipitation with IkB α antibodies. Following SDS-PAGE of the precipitate, the labeled proteins were visualized by autoradiography and quantified by phosphoimager. The half-lives of the different proteins are indicated.

ples were relatively equal. The stability of these proteins in vivo was determined in a pulse-chase experiment in which the transfected cells were labeled for 1 h and then incubated for 2 h in the absence of cold methionine-cysteine. Equal aliquots were taken at 0, 30, 60, and 120 min following the chase, and the labeled IkB α proteins were immunoprecipitated and separated by SDS-PAGE (Fig. 5B). A quantitative analysis using phosphoimager enabled us to determine the half-life of the IkB α proteins. As can be seen in Fig. 5B, MutF has a half-life twice as long as IkB α (32 min versus 71 min). Interestingly, an aspartic acid substitution at position 293 can completely revert the MutF phenotype. A serine substitution at position 293

also correct the MutF phenotype. We interpret these results, taken together, to mean that the constitutive phosphorylation of the C-terminal region of IkB α by CKII is necessary for the rapid proteolysis of free IkB α .

DISCUSSION

Multicellular organisms have developed signal transduction pathways important for differentiation and developmental programs as well as for immunologic defense responses. The NF- κ B/Rel signal transduction pathway is relied upon extensively in both invertebrate and vertebrate animals for these purposes (5, 28, 33, 36). Perhaps a main salient feature of this pathway is the rapidity with which NF- κ B/Rel proteins become activated and enable cells to respond to external signals instantaneously. Although the complete mechanism of NF- κ B signal transduction is not yet known, it is clear that the pivotal event is the signal-induced phosphorylation of the N terminus of IkB α , leading to its degradation and subsequent NF- κ B activation (1, 18, 26, 35).

The C-terminal region of IkB α contains a PEST region which may be involved in the degradation process (11, 29). While PEST sequences are used in a large number of proteins which are regulated at the level of stability (30), it is still not known what constitutes a functional PEST and how it facilitates protein degradation. To understand the mechanism by which IkB α is rapidly degraded, we have investigated its constitutive phosphorylation and found that it is an integral part of the PEST. We have mapped the constitutive phosphorylation site to serine 293, which places it right in the middle of the PEST (Fig. 1). We also show that the constitutive kinase, CKII, is capable of phosphorylating several residues in the PEST region and that compensatory phosphorylation can occur (Fig. 2 through 4). The fact that the multiple CKII phosphorylation sites in the IkB α PEST region are conserved (Fig. 1) favors this notion.

One of the first genes whose transcription is induced upon NF- κ B activation is IkB α (15). The newly synthesized IkB α protein is constitutively phosphorylated at serine residue 293 by CKII. The CKII phosphorylation site resides in the PEST region identified in IkB α (9). PEST sequences are rich in P, E/D, S, and T and are thought to be associated with rapid protein turnover (30). Thus, it is reasonable to assume that posttranslational modification of the PEST sequence may influence its role in protein turnover. Indeed, we have shown that IkB α -MutF protein (where all five possible CKII phosphorylation sites are mutated to Ala) is more stable than wild-type IkB α (Fig. 5B).

The IkB α protein present in the cell is phosphorylated and can conceivably "choose" at least three fates: (i) association with p50/RelA complexes to sequester them in the cytoplasm, (ii) transport to the nucleus, where it can bind to p50/RelA or other NF- κ B complexes and dissociate them from bound DNA (this could be a very efficient mechanism of downregulation of NF- κ B activity; it is not clear if the fraction of IkB α that traverses to the nucleus is phosphorylated), and (iii) degradation, i.e., the excess IkB α that is not in association with any NF- κ B complexes is slated for degradation. In the first case where IkB α is associated with NF- κ B protein, it undergoes signal-induced degradation to allow NF- κ B proteins to translocate to the nucleus. This degradation requires an intact N-terminal which is hyperphosphorylated at serine residues 32 and/or 36, ubiquitinated, and then degraded by proteasomes (14). The signal-induced degradation is independent of the PEST sequences, since IkB α Δ 39 (where 39 amino acids at the C terminus containing PEST sequences are deleted) is de-

graded in response to phorbol myristate acetate, LPS, or TNF- α (35a). It is, therefore, unlikely that constitutive phosphorylation of I κ B α plays a significant role in the signal-induced degradation of I κ B α .

What about the excess I κ B α ? How does free I κ B α undergo degradation? Our data show (Fig. 5B) that free I κ B α which is not associated with NF- κ B proteins is degraded with a half-life of about 32 min (Fig. 5B), as compared with less than 2 to 5 min in the case of TNF- α induced degradation of NF- κ B-bound I κ B α (20). If the CKII phosphorylation sites are mutated, the half-life of free I κ B α is doubled to nearly 71 min. It seems that phosphorylation of Ser-293 alone is perhaps sufficient for rapid degradation of free I κ B α . Furthermore, if Ser-293 is mutated to Asp-293, which provides the acidic charge, the half-life is equal to that of the free I κ B α (Fig. 5B). The half-life of I κ B α is not influenced by N-terminal residues Ser-32 and Ser-36 (data not shown), which have been shown to be essential for induced degradation of I κ B α bound to NF- κ B proteins (11). We therefore conclude that modification of PEST sequences by phosphorylation is an integral part of a mechanism required for degradation of free I κ B α protein.

ADDENDUM

Since the completion of this work, three other groups have identified CKII as the constitutive, C-terminal I κ B α kinase (22–24).

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