THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 28, Issue of July 14, pp. 21278–21286, 2000

Printed in U.S.A.

Signal-dependent and -independent Degradation of Free and NF-κB-bound IκBα*

Received for publication, March 24, 2000, and in revised form, April 28, 2000
Published, JBC Papers in Press, May 2, 2000, DOI 10.1074/jbc.M002532200

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A family of inhibitory IκB molecules regulates the activation of the transcription factor NF-κB. One member of the IκB family, IκBα, plays a major role in the rapid signal-induced activation of NF-κB. IκBα itself is transcriptionally regulated by NF-κB allowing for a tight autoregulatory loop that is both sensitive to and rapidly influenced by NF-κB activating stimuli. For this pathway to remain primed both for rapid activation of NF-κB in the presence of signal and then to suppress NF-κB activation once that signal is removed, IκBα must be exquisitely regulated. The regulation of IκBα is mainly accomplished through phosphorylation, ubiquitination, and subsequent degradation. The mechanism(s) that regulate IκBα degradation needs to be able to target IκBα for degradation in both its NF-κB bound and free states in the cell. In this study, we utilize a full-length IκBα mutant that is unable to associate to RelA/p65. We show that the signal-induced IκB kinase (IKK) phosphorylation sites on IκBα can only significantly influence the regulation of signal-dependent but not signal-independent turnover of IκBα. We also demonstrate that the constitutive carboxyl-terminal casein kinase II phosphorylation sites are necessary for the proper regulation of both signal-dependent and -independent turnover of IκBα. These findings further elucidate how the phosphorylation of IκBα influences the complex regulatory mechanisms involved in maintaining a sensitive NF-κB pathway.

The transcription factor NF-κB is an important regulator of genes involved in immune and inflammatory responses, apoptosis, and cell proliferation (1, 2). The NF-κB/Rel family of transcription factors are a family of proteins that homo- and heterodimerize through a conserved Rel homology domain that consists of approximately 300 amino acids. The Rel homology domain of NF-κB is responsible for homo- and heterodimerization, DNA binding activity, and nuclear localization (3–5). A large number of stimuli can cause NF-κB to translocate from the cytoplasm to the nucleus, and activate target gene transcription. Stimuli that can activate NF-κB include proinflammatory cytokines, bacterial lipopolysaccharide, phorbol esters, okadaic acid, and viral infection (2, 6–8).

A group of inhibitory proteins belonging to the IκB family regulate NF-κB activation by sequestering NF-κB in the cytoplasm. IκB exerts its inhibitory effects by associating with the Rel homology domain of NF-κB proteins, effectively masking their nuclear localization signal (9–12). Although there are a number of IκB proteins, IκBα is the primary regulator of rapid signal induced activation of NF-κB. Upon stimulation by a proinflammatory cytokine such as TNFα,[1] a signaling cascade is initiated that results in the activation of the IκB kinases IKK1 and IKK2 (13–17). This leads to the rapid phosphorylation of IκBα at the signal-induced phosphorylation sites, serine 32 and serine 36 (18–21). Once phosphorylated, IκBα is polyubiquitinated by the Ubc5/E3RS$^{I\kappa B}$ ubiquitination enzyme pair (22, 23) on lysine 21 and lysine 22 (24–26). Polyubiquitinated IκBα is degraded by the 26 S proteasome, thus exposing NF-κB's nuclear localization signal and allowing NF-κB to translocate to the nucleus (27–29). Once in the nucleus NF-κB activates transcription of target genes including IκBα (6, 30). When the NF-κB inducing signal is removed the newly synthesized IκBα can suppress NF-κB activity by preventing it from binding to the genomic DNA, and sequestering NF-κB in the cytoplasm (31–33).

The regulation of IκBα is carried out mainly through phosphorylation. Several phosphorylation sites have been identified on IκBα and they include the signal-induced IKK phosphorylation sites located at serine 32 and serine 36 (18–21), the constitutive CKII phosphorylation sites located in the carboxyl-terminal PEST domain (34, 35), the protein kinase C site located in Ankyrin repeat 6 (ank6) (36, 37), and a tyrosine phosphorylation site that can cause the dissociation but not the degradation of IκBα from NF-κB in Jurkat T cells (38). Although extensive analysis of the signal-dependent degradation of IκBα has been done, little distinction has been made between the signal-dependent and -independent degradation of free and NF-κB-bound IκBα. Therefore, we undertook a study to examine the role of various phosphorylation sites on IκBα to determine their effects on both the signal-dependent and -independent degradation of free and NF-κB-bound IκBα.

In this study, we characterize a full-length IκBα mutant that is unable to associate with NF-κB (designated as mutC) and exists as a free molecule in the cell. We also show that distinct phosphorylation sites can directly influence the efficiency of ubiquitination and subsequent degradation of free and NF-κB-associated IκBα in the presence or absence of stimuli. These results demonstrate the complexity of IκBα regulation necessary to ensure that NF-κB is rapidly and specifically activated by a diverse group of stimuli.

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¶ Graduate student supported by consecutive fellowships from the San Diego Fellowship and the Chapman Charitable Trust.

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[1] The abbreviations used are: TNFα, tumor necrosis factor α; CKII, casein kinase II; MEF, mouse embryo fibroblasts; IP, immunoprecipitation.

EXPERIMENTAL PROCEDURES

Materials—The proteasome inhibitor *clasto*-lactacystine β-lactone (β-lactone) and the ubiquitin hydrolase inhibitor ubiquitin aldehyde (Ubal) were purchased from Boston Biochem Inc. Ubiquitin and cyclohexamide were purchased from Sigma and okadaic acid was purchased from Life Technologies, Inc. Antibodies against IκBα (c-21, sc-371), RelA/p65 (sc-109), and IKKβ (H-470) were purchased from Santa Cruz Biotechnology. Antibodies against the FLAG (M2) and HA (12CA5) tags were purchased from Eastman Kodak Co. and Roche Molecular Biochemicals, respectively. TNFα, inorganic pyrophosphatase, and creatine phosphokinase were purchased from Calbiochem.

Cell Culture—HeLa cells, human embryonic kidney 293 cells, and IκBα −/− mouse embryo fibroblasts (MEF) (39) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in an atmosphere of 10% CO_2, at 37 °C. 293 cells stably expressing Moloney *gag* and *pol* (293gp) were maintained as above and selection was maintained with blastacidin (20 μg/ml). TNFα and β-lactone were used at final concentrations of 10 ng/ml and 10 μM, respectively, unless otherwise noted. Cyclohexamide was used at a final concentration of 75 μg/ml.

Plasmids and in Vitro Translation—Mutants were generated by polymerase chain reaction-based site-directed mutagenesis and confirmed by sequencing. Wild-type (wt) murine IκBα, mutF (S283A, S288A, T291A, S293A, and T296A), 3236 (S32A and S36A), M (mutF and 3236 mutations combined), mutC (T247A, S252A, T257A, S262A, and T263A), and 3236mutC (3236 and mutC mutations combined) were ligated into the *Bam*HI and *Hin*dIII sites of the pCMX-PL1 polylinker (40). PCMX-RelA/p65 was described previously (30). Wild-type IκBα and the IκBα mutants were also ligated into the pCLBabepuro retroviral vector. pCLBabepuro is a derivative of the retroviral construct pBabepuro (41). The pCMX-PL1-IκBα constructs and pCLBabepuro were cut with *Hin*dIII and *Eco*RI, respectively, and then blunted with Klenow. Both were then cut with *Bam*HI and the resulting *Bam*HI/*Hin*dIII(blunt) IκBα fragment was ligated into the *Bam*HI/*Eco*RI(blunt) pCLBabepuro retroviral vector.

In vitro transcription-translation was performed using the T7 promoter contained in the PCMX-PL1 vectors. Wild-type and mutant IκBα proteins labeled with [^{35}S]methionine were produced using a wheat germ extract TNT kit as instructed by the manufacturer (Promega). 60 μl of the TNT reaction was used for each sample that was tested in the *in vitro* ubiquitination assay.

Transfections—293 cell transfections were performed by the calcium phosphate method (42). Briefly, DNA was mixed in 0.625 M $CaCl_2$ and then added to an equal volume of 2 × HEPES buffer saline solution (560 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4, pH 7.1). The mixture was added to approximately 10^6 cells in 10 ml of medium and incubated at 37 °C for 6 h (h) in 3% CO_2. The medium was then changed and the cells were incubated for 48 h at 37 °C, in 10% CO_2.

Production of Virus and Stable Pools—Virus production for infection of the IκBα −/− MEF cell line was performed by calcium phosphate co-transfection of the 293gp packaging cell line with 20 μg of the pCLBabepuro retroviral vector, containing cDNAs for wild-type (wt) IκBα or the various IκBα mutants, and 5 μg of the pMDG plasmid containing the vesicular stomatitis virus (VSVg) envelope downstream of the cytomegalovirus promotor-enhancer. After 48 h, the media was removed, filtered, Polybrene added (8 μg/ml), and it was immediately used for infection. Each infection was performed by adding 3 ml of 1:1000 diluted virus supernatant to approximately 10^5 cells for 6 h. The cells were allowed to expand for 48 h and the infected cells were selected using 6 μg/ml puromycin (Calbiochem). In order to get equivalent levels of expression in the mutC and 3236mutC stable pools it was necessary to perform three rounds of infection and selection as described above.

Cell Stimulation and Western Blot Analysis—The IκBα −/− MEF stable pools were stimulated with 10 ng/ml TNFα for the given time points. In some cases the MEFs were pretreated with 10 μM β-lactone, for 1 h, prior to stimulation. MEFs were also treated with β-lactone for the indicated times in the absence of signal. Cells were then washed 2 times with ice-cold phosphate-buffered saline and frozen on dry ice. Cytoplasmic extracts were made as described previously and separated on 10 or 12% SDS-polyacrylamide gels and transferred to 0.2-μm nitrocellulose membranes (Schleicher & Schuell). Membranes were probed with antibody, diluted 1:1,000 in 0.2% Tween-phosphate-buffered saline containing 5% nonfat milk, for 4 h at 4 °C. Horseradish peroxidase-conjugated donkey anti-rabbit sera (Amersham Pharmacia Biotech) was diluted 1:3,000 and incubated with the membranes for 1 h at room temperature. Specific bands were then resolved by using a Renaissance detection kit (NEN Life Science Products Inc.) as instructed by the manufacturer.

Electrophoretic Mobility Shift Assay—Nuclear extracts were made by the micropreparation technique (43), and gel shift analysis was performed as described previously (44). Briefly, 5 μg of nuclear protein extract was incubated with 0.5 μg of poly(dI-dC), on ice, for 20 min to block nonspecific DNA binding activity. A ^{32}P-labeled oligonucleotide containing the HIV-1 long terminal repeat κB site was then added, and the mixture was incubated at room temperature for 30 min. The resulting complexes were then resolved on a 4% acrylamide gel, exposed to a PhosphorImager (Molecular Dynamics), and band intensities quantitated with ImageQuant software.

In Vitro Ubiquitination Assay—HeLa cytoplasmic extracts, used in the *in vitro* ubiquitination assay, were made by lysis in hypotonic buffer (buffer A) containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride and 10 μg/ml aprotinin). Lysates were cleared by centrifugation at 14,000 rpm, for 15 min, at 4 °C. Lysates were then concentrated with a Centricon 3 concentrator (Amicon) as instructed by the manufacturer. Protein concentration was determined using the Bio-Rad protein assay system.

In vitro ubiquitination assays were carried out as described (29) with the following changes. Briefly, *in vitro* ^{35}S-labeled IκBα was incubated with 5 mg/ml HeLa extract for 15 min at 4 °C. A reaction mixture containing 50 mM Tris (pH 7.5), 5 mM $MgCl_2$, 2 mM ATP, 10 mM creatine phosphate, 3.5 units/ml creatine kinase, 0.6 units/ml inorganic pyrophosphatase, 1 mg/ml ubiquitin, 3 μM okadaic acid, 3 μM ubiquitin aldehyde, and 10 μM β-lactone was then added and the reaction was incubated at 37 °C for 90 min. 50 mM Tris (pH 7.5) was substituted for the reaction mixture in control samples.

After the incubation, RIPA (100 mM NaCl, 20 mM Tris, pH 8.0, 0.5% Nonidet P-40) containing 5 mM *N*-ethylmaleimide was added and the samples were subjected to immunoprecipitation (IP) by antibodies directed against IκBα or RelA/p65. IPs were carried out with 1.5 μg of the appropriate antibody for 1 h at 4 °C. Immunocomplexes were precipitated by incubation with protein A-Sepharose (Amersham Pharmacia Biotech) for 1 h at 4 °C. The pellets were washed in RIPA buffer, SDS sample buffer was added, and the pellets were boiled for 5 min prior to SDS-polyacrylamide gel electrophoresis on 10% gels. Following electrophoresis, the gels were fixed in glacial acetic acid, amplified with 2,5-diphenyloxazole, rinsed with H_2O, dried, and exposed to a PhosphorImager for quantification.

Metabolic Labeling—6-cm plates of the IκBα −/− MEF stable pools were washed twice in phosphate-buffered saline and incubated for 1 h in 2 ml of cystine- and methionine-free Dulbecco's modified Eagle's medium supplemented with 10% dialyzed fetal bovine serum (Life Technologies, Inc.). Labeling was performed for 3 h with 0.5 mCi of [^{35}S]methionine (NEN Life Science Products Inc.) per ml. Cells were then washed three times with complete medium and then chased for the given time points in complete medium. After each time point the cells were washed twice with ice-cold phosphate-buffered saline and cell pellets were frozen on dry ice for later manipulation. Cell pellets were thawed on ice and whole cell lysis was performed by adding 500 μl of RIPA (20 mM Tris, pH 8.0, 100 mM NaCl, 0.2% sodium deoxycholate, 0.2% Nonidet P-40, 0.2% Triton X-100) containing the protease inhibitors aprotinin (10 μg/ml; Sigma) and phenylmethylsulfonyl fluoride (1 mM; Sigma). DNA was sheared by 10 passes through a 20-gauge needle and lysates were cleared by centrifugation at 14,000 rpm for 15 min, at 4 °C. The cleared lysates were measured for trichloroacetic acid-precipitable counts and the lysates were normalized for labeling efficiency. Normalized lysates were precleared with protein A-Sepharose for 1 h at 4 °C and then incubated with RelA/p65 antiserum plus protein A-Sepharose for 4 h at 4 °C. To reduce the background, coimmunoprecipitations that were done with the RelA/p65 antiserum were subjected to a second round of immunoprecipitation. The RelA/p65 antibody protein A-Sepharose pellets were boiled for 10 min in RIPA containing 100 μg of bovine serum albumin/ml and 0.5% SDS. The SDS was diluted to 0.1% with RIPA buffer that contained 100 μg of bovine serum albumin. A second IP was then done by adding IκBα antiserum and protein A-Sepharose to isolate the RelA/p65-associated IκBα. To isolate the free IκBα, the supernatant from the original Rela/p65 co-IP was incubated with IκBα antiserum and protein A-Sepharose. After immunoprecipitation, the pellets were washed in RIPA buffer, resuspended in SDS sample buffer, boiled for 5 min, and the eluted proteins were separated on a 12% SDS-polyacrylamide gel.

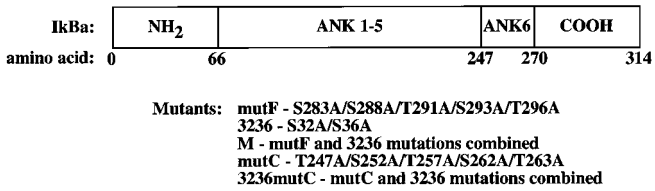

FIG. 1. **Schematic display of the IκBα mutants.** *MutF* is a mutation of all of the carboxyl-terminal CKII phosphorylation sites. *3236* is a mutation of the amino-terminal IKK sites. *M* is a combination of the mutF mutation and the 3236 mutation. *MutC* is a mutation of all the possible phosphorylation sites in the ank6 region. 3236mutC is a combination of the 3236 mutation and the mutC mutation.

RESULTS

Signal-dependent Degradation of IκBα—To investigate the signal-dependent degradation of IκBα *in vivo*, stable pools of IκBα −/− MEFs (39) were generated by infecting naive IκBα −/− MEFs with recombinant retroviral vectors containing either wild type (wt) murine IκBα, one of the IκBα mutants (Fig. 1 schematically describes IκBα mutations), or GFP. The mutants described in Fig. 1 represent mutations of known and possible sites of serine and threonine phosphorylation. These mutants were chosen for this study because they would aid in the further elucidation of the role of serine and threonine phosphorylation in the signal-dependent and -independent degradation mechanisms of free and NF-κB-bound IκBα. The IκBα −/− MEF infections were carried out at a multiplicity of infection of much less than one to ensure that on average, after selection, each cell of a stable pool would have only a single copy of the integrated recombinant retroviral vector. This kept expression levels low and as close to endogenous expression levels as possible. Fig. 2*A* compares the relative expression levels of endogenous IκBα in wt MEF cells to that of the transduced IκBα −/− stable pools. It can be seen that the levels of the IκBα proteins in the transduced cells are within 1–2-fold of the endogenous IκBα (Fig. 2, *lane 8*, +/+).

TNFα stimulation of the IκBα −/− MEF stable pools in the presence of the translational inhibitor cyclohexamide show that, compared with wild type IκBα, the 3236 and M mutants were significantly more stable (Fig. 2*B*). The M mutant shows no change in expression over the time course while the 3236 mutant starts to show a slight reduction at the 60-min time point. The mutF mutation gives IκBα partial resistance to TNFα-dependent degradation but is less stable than the 3236 and M mutants. The mutC mutant shows no increase in stability and, in fact, is significantly less stable than wt IκBα. The 3236mutC mutation confers an increase in the stability of mutC containing IκBα, but is significantly more unstable than the 3236 mutation by itself.

The rate of IκBα degradation shown in Fig. 2*B* was further examined by measuring the NF-κB DNA binding activity following TNFα stimulation. The mutF mutation shows an approximate 50% reduction in DNA binding activity when compared with wt IκBα (Fig. 2*C*). The 3236 and M mutations reduce the gel shift activity to what is observed in unstimulated control cells. Interestingly, cell pools containing either the mutC or 3236mutC mutations show no significant reduction in NF-κB DNA binding activity. This is most likely due to the fact that the mutC mutation, which is a substitution of all five serine and threonine residues in the ank6 region of IκBα to alanine, disrupts the association of IκBα to NF-κB. Fig. 3, *A* (*in vitro*) and *B* (*in vivo*), show that both mutC and 3236mutC do not associate with RelA/p65. It was recently shown that the ank6 region of IκBα makes a critical contact to NF-κB (11, 12). Since the mutC mutation disrupts IκBαs association to NF-κB and blocks any potential phosphorylation of ank6, it is possible that phosphorylation of ank6 plays a critical role in IκBα's association to NF-κB. We conclude that (i) IKK phosphorylation is necessary for signal-dependent degradation of both free and NF-κB-associated IκBα, and (ii) that CKII phosphorylation is necessary for efficient signal-dependent degradation of NF-κB-associated IκBα.

Next, *in vitro* ubiquitination assays were performed to determine the signal-induced ubiquitination profiles of both free and NF-κB-associated IκBα mutants. A representative gel is shown for each experiment. Quantitation was done on a PhosphorImager, using ImageQuant software, and the resulting histograms display the percentage of the total labeled IκBα in the reaction that was shifted. By graphing the percentage of the total IκBα shifted we were able to correct for any loading differences between samples. Therefore, the histograms give a more accurate representation of the respective levels of ubiquitination on the various IκBα molecules. When examining a pool of both RelA/p65-associated and free IκBα it appears that IκBα containing the mutF mutation is ubiquitinated slightly, but reproducibly, more efficiently than wt IκBα (see Fig. 4*A, lanes 1* and *2*). The level of ubiquitination of the other IκBα mutants in Fig. 4*A* is consistent with the rate of degradation and the gel shift results presented in Fig. 2, *B* and *C*. The 3236 and M mutants have levels of ubiquitination just above background (Fig. 4*A, lanes 3* and *4*).

The data with the mutC mutants (Fig. 4*A, lanes 5* and *6*) represents the levels of ubiquitination on free IκBα molecules, since mutC does not associate with NF-κB (Fig. 3, *A* and *B*). MutC alone is ubiquitinated very efficiently (*lane 5*), even better than wt (Fig. 4*A, lane 5,* compared with *lane 1*, respectively). The mutC3236 mutant shows a reduction in the level of ubiquitination compared with mutC alone, but the level of ubiquitination of 3236mutC is significantly higher than IκBα containing just the 3236 mutation (Fig. 4*A, lane 6,* compared with *lane 3,* respectively). These data demonstrate that free IκBα does undergo signal-induced phosphorylation and ubiquitination and that serine 32 and serine 36 play a significant role in this process.

Fig. 4*B* displays the ubiquitination of the RelA/p65 associated pool of IκBα. The mutF mutation reduces the amount of signal-dependent ubiquitination below the level seen on wt IκBα when it is in association with NF-κB (Fig. 4*B, lanes 1* and *2*). This reduction in ubiquitination by mutF of only NF-κB-associated IκBα explains why IκBα is partially stabilized and why a reduction in the DNA binding activity of NF-κB is observed in the IκBα −/− MEF mutF stable pool (Fig. 2, *B* and *C*).

In contrast to the NF-κB-bound IκBα (Fig. 4*B, lanes 1* and *2*), the signal-induced ubiquitination of only the free pool of IκBα reveals that mutF is ubiquitinated slightly more efficiently than the wt (Fig. 4*C, lanes 1* and *2*). The 3236 and M mutants give large reductions in the amount of ubiquitination observed (Fig. 4*C, lanes 4* and *5*). Ubiquitination of mutC and 3236mutC, in the *in vitro* ubiquitination assay, could only be observed by direct anti-IκBα immunoprecipitation (Fig. 4*A, lanes 5* and *6*) because these mutants do not associate to RelA (Fig. 3*A*). The *in vitro* ubiquitination assay data (Fig. 4, *A-C*) correlates well with the stability and gel shift observations made in the IκBα −/− MEF stable pools (Fig. 2, *B* and *C*).

The *in vitro* ubiquitination results were confirmed by performing *in vivo* ubiquitination assays in the stably transduced IκBα −/− MEF stable pools. The IκBα −/− MEF stable pools were first treated with β-lactone, a potent and specific inhibitor of the proteasome, and then stimulated with TNFα (Fig. 4*D*). The ubiquitination patterns obtained in this experiment are similar to the corresponding in the *in vitro* assays. Wt, mutF,

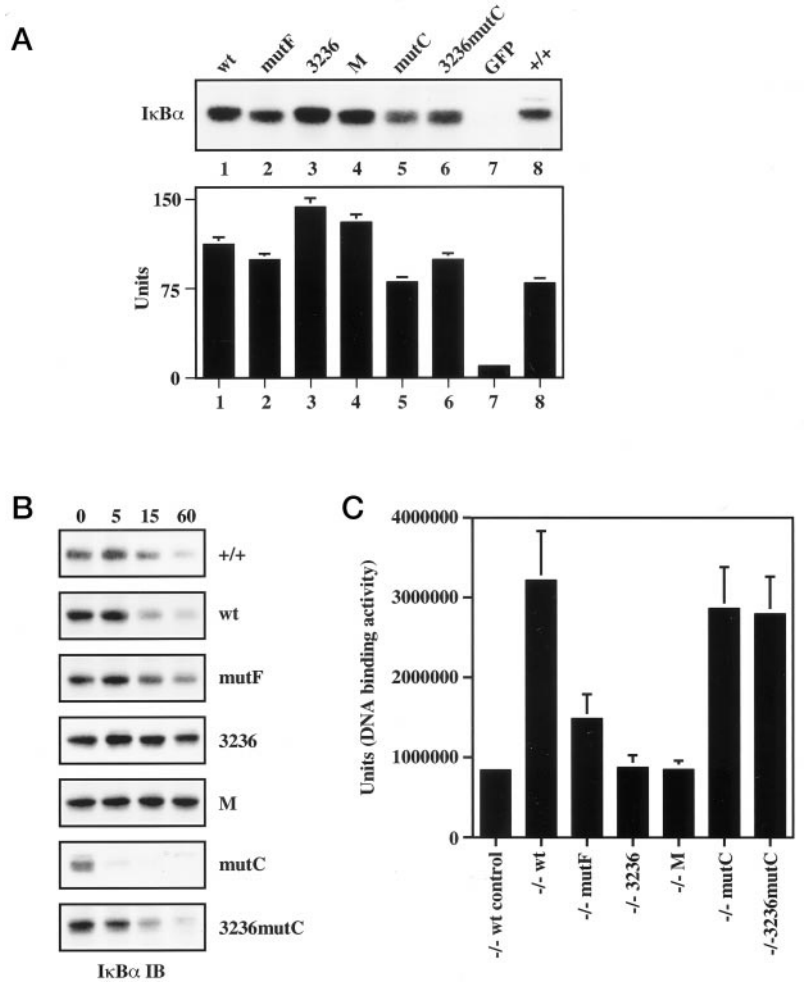

FIG. 2. **Signal-dependent activation of IκBα −/− MEF stable pools.** *A,* IκBα immunoblot (*IB*) of the relative expression levels of the various IκBα −/− stable pools, an IκBα −/− GFP stable pool, and endogenous IκBα expression in IκBα +/+ MEFs. Quantitation of the relative expression level of IκBα in each cell line was done using NIH Image 1.62 software. The results are given graphically in arbitrary units. *B,* IκBα −/− MEFs were infected with a recombinant retroviral vector containing wild type murine IκBα or one of the mutants. The stable pools were treated with cyclohexamide prior to being stimulated with TNFα for 0, 5, 15, or 60 min. Following stimulation the cells were harvested, cytoplasmic extracts made, and Western blot analysis was performed with anti-IκBα sera. *C,* gel shift analysis of IκBα −/− MEF stable pools stimulated with TNFα for 60 min. Three independent experiments were done. Quantification was done on a PhosphorImager, using ImageQuant software. The values obtained were averaged and the standard deviations were calculated.

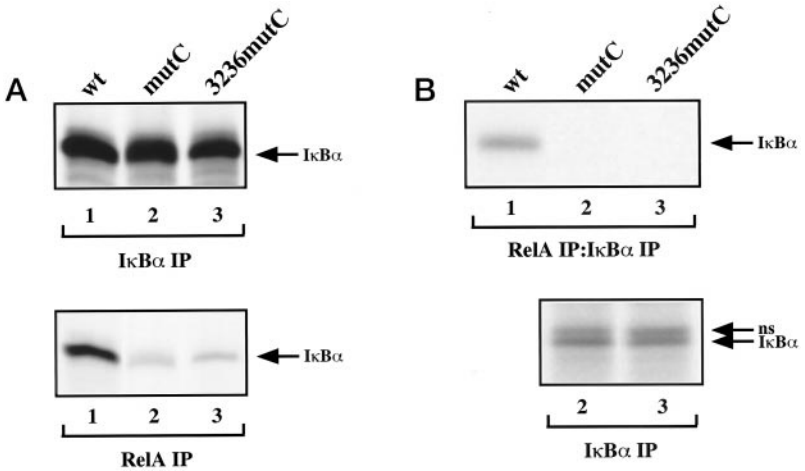

FIG. 3. **Association of wild type and mutC containing IκBα mutants to RelA/p65 *in vitro* and *in vivo*.** *A, in vitro* association assay. ^{35}S-Labeled IκBα, mutC, or 3236mutC were mixed with uninduced cytoplasmic extract as described under "Experimental Procedures." The sample was then immunoprecipitated with anti-IκBα or anti-RelA. The IκBα IP shows input amount of labeled IκBα species. The RelA IP shows the amount of the different IκBα species that associated to the RelA/p65 present in the cytoplasmic extract. *B, in vivo* association assay. The wild type, mutC, and 3236mutC IκBα −/− stable pools were labeled with [^{35}S]methionine for 3 h. The cells were washed and then lysed in RIPA buffer. A RelA IP was performed and then the precipitated material was subjected to a second round of IP with anti-IκBα sera to reduce the background. This was undertaken to examine at RelA/p65-associated IκBα in these cell lines. IκBα IPs were also done to show that IκBα is being expressed and labeled in the mutC and 3236mutC stable pools.

3236, and M represent NF-κB associated IκBα (Fig. 4*D, lanes 1–4*, respectively) and give ubiquitination profiles that match those obtained for RelA/p65-associated IκBα in the *in vitro* assay (Fig. 4*B, lanes 1–4*). Wt IκBα is efficiently ubiquitinated in the presence of signal, mutF causes a decrease in ubiquitination, and 3236 and M reduce ubiquitination to almost background levels (Fig. 4*D, lanes 1, 2, 3*, and *4,* respectively).

MutC and 3236mutC represent free IκBα in the IκBα −/− MEF stable pools (Fig. 3*B*), and they also give ubiquitination patterns *in vivo* (Fig. 4*D, lanes 5* and *6*) that correlate with

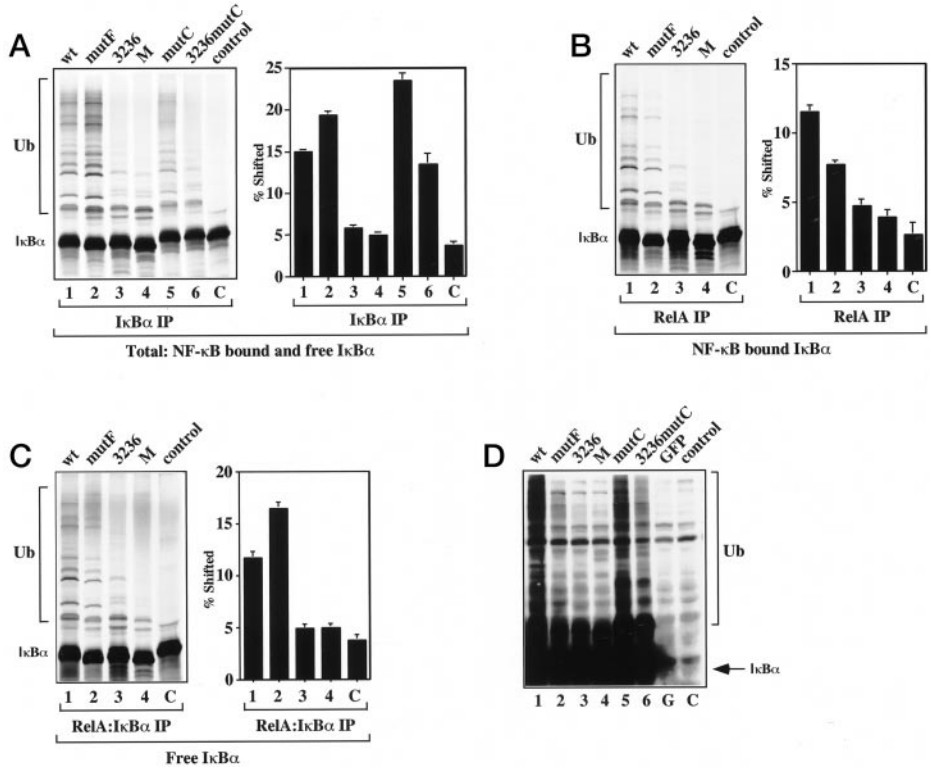

FIG. 4. ***In vitro* and *in vivo* signal-dependent ubiquitination of the IκBα mutants.** All experiments in this figure were done under stimulated conditions. The HeLa cytoplasmic extracts used in the *in vitro* ubiquitination assays were activated with a reaction mixture that contained 3 μM okadaic acid. Control samples, designated by "C," were performed exactly like the wt sample except the reaction mixture was replaced by 50 mM Tris (pH 7.5). *A,* the *in vitro* signal-dependent ubiquitination assays were carried out as described under "Experimental Procedures." The IκBα IP was carried out to examine total ubiquitination of both free and NF-κB-associated IκBα. Quantitation was done on the PhosphorImager, using ImageQuant software. The percentage of labeled IκBα shifted by ubiquitination was calculated by dividing the shifted counts by the total count. This calculation corrects for any differences in the input amounts of labeled IκBα. Three independent samplings were performed and the average of the three values obtained is depicted graphically. *B,* the RelA IP was done to isolate the labeled IκBα that had become associated to NF-κB present in the activated HeLa extract. Everything else is as was done for *A*. *C,* the supernatant from the RelA IP done in *B* was subjected to a second round of IP with anti-IκBα sera. This isolated the free IκBα in the *in vitro* ubiquitination assay. *D, in vivo* signal-dependent ubiquitination. The IκBα −/− MEF stable pools were treated with β-lactone for 1 h and then stimulated with TNFα for 15 min. Cytoplasmic extracts were made and an IκBα immunoblot was performed. *Ub*, ubiquitin.

those obtained *in vitro* (Fig. 4*A, lanes 5* and *6*). MutC alone is ubiquitinated very efficiently and the 3236mutC mutation gives a reduction in the amount of ubiquitination but the level of ubiquitination seen is significantly higher than that seen with just the 3236 mutant (Fig. 4*D*, compare *lanes 3* and *6*). These data show that free IκBα is ubiquitinated in the presence of signal even when it is lacking its signal-dependent phosphorylation sites. It is likely that signal-independent degradation mechanisms that are involved in removing free IκBα from the cell are contributing to the ubiquitination of the mutC containing mutants in these experiments.

From the *in vitro* and *in vivo* ubiquitination data we conclude that phosphorylation of serine 32 and serine 36 by the IKK is necessary for efficient signal-dependent ubiquitination and degradation of free and NF-κB-associated IκBα. We also conclude that the constitutive CKII phosphorylation of the carboxyl terminus of IκBα enhances the extent of ubiquitination of free IκBα (Fig. 4*C, lane 2*), but reduces the ubiquitination of NF-κB associated IκBα (Fig. 4, *B* and *D, lane 2*).

Signal-independent Turnover of IκBα—To investigate whether or not the constitutive or signal-dependent phosphorylation sites are involved in the signal-independent turnover of NF-κB-associated IκBα, pulse-chase experiments were performed on the wt, mutF, and 3236 IκBα −/− MEF stable pools (Fig. 5, *A-C*). Compared with the half-life of wt IκBα, the mutF mutation gives a 2-fold increase in IκBαs half-life. The 3236 mutation has no effect on basal turnover, resulting in a half-life very similar to that of wt IκBα (compare *A* and *C*). These results demonstrate that only the carboxyl-terminal CKII phosphorylation sites play a significant role in the basal turnover of NF-κB-associated IκBα. The inducible phosphorylation sites (serine 32 and 36) have no effect on the basal turnover of NF-κB-bound IκBα.

Several studies have been performed to evaluate the role of phosphorylation in the degradation of free IκBα in the absence of stimulation (35, 45, 46). It has been shown that the mutF mutation or the removal of the carboxyl terminus of IκBα will increase the stability of free IκBα by approximately 2-fold (35, 46). Here we look at the effects of the 3236 mutation on the basal turnover of free IκBα. Unstimulated IκBα −/− MEF stable pools expressing either the mutC mutant or the 3236mutC mutant (Fig. 6, *A* and *B*) were analyzed. As has been shown (Fig. 3*B*) mutC and 3236mutC do not associate to RelA/p65, thus all the IκBα is in a free state in the IκBα −/− MEF stable pools. Free IκBα is at least 5 times more rapidly degraded in the absence of signal when compared with NF-κB-bound IκBα (compare Fig. 5*A* and 6*A*). Interestingly, the 3236 mutation in the mutC background increases the rate of signal-independent turnover of free IκBα by about 3-fold (Fig. 6, *A* and *B*).

To determine whether or not ubiquitination plays a role in the basal turnover of NF-κB-associated IκBα, the IκBα −/− MEF stable pools were treated with the proteasome inhibitor β-lactone in the absence of signal. Unfortunately, the prolonged treatments with β-lactone necessary to see the accumulation of ubiquitinated IκBα bound to NF-κB in the absence of signal seems to activate the NF-κB pathway (data not shown). This is

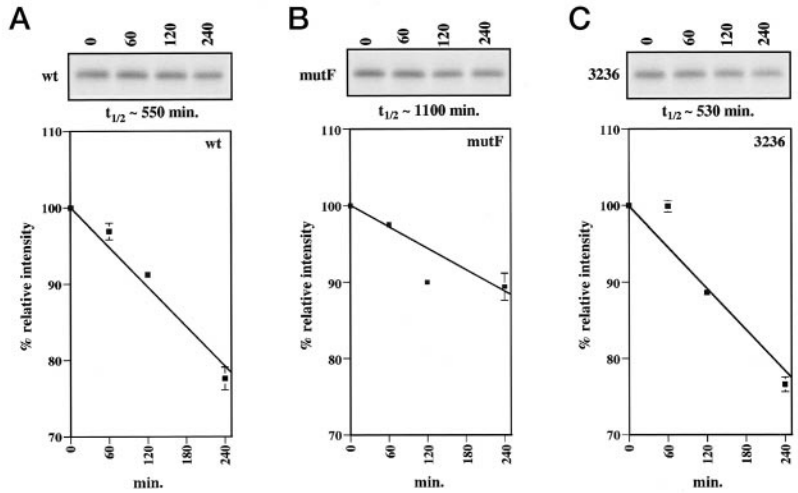

FIG. 5. **Signal-independent turnover of NF-κB-bound IκBα.** *A-C,* pulse-chase analysis was performed to determine the half-life of NF-κB-associated IκBα under signal-independent conditions. The stable pools were metabolically labeled with [^{35}S]methionine for 3 h, washed, and then chased for 0, 60, 120, or 240 min. A RelA IP was performed and then the precipitated material was subjected to a second round of IP with anti-IκBα sera to reduce the background. This isolated the RelA/p65-associated IκBα in these cell lines. Quantitation was done on the PhosphorImager, using ImageQuant software. The data were converted into percent relative intensity by assigning the zero time point of each time course as 100% and assigning the remaining time points a percentage that correlates to the fraction of its signal. The percent relative intensities were then graphed against time, in minutes, and half-lives were calculated.

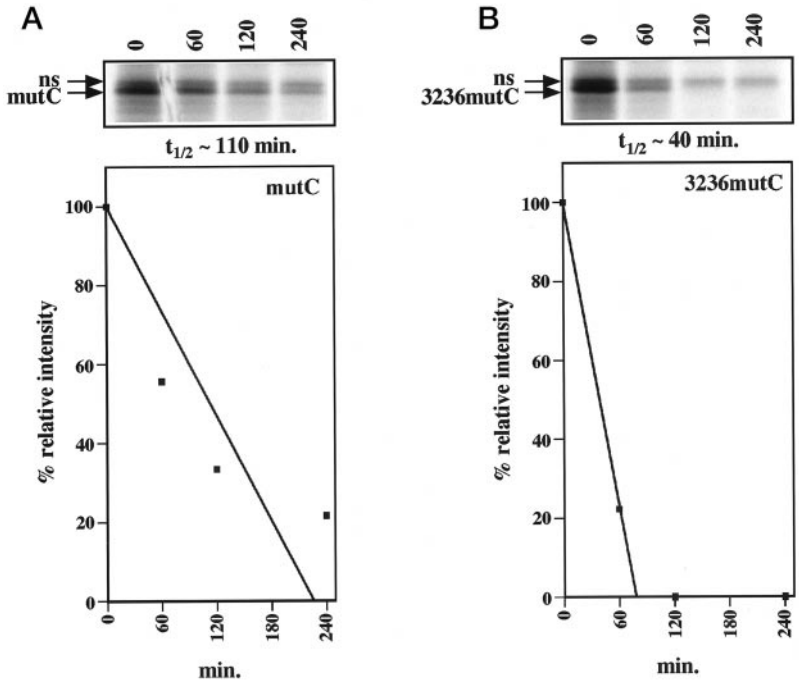

FIG. 6. **Signal-independent turnover of free IκBα.** *A* and *B,* everything was done the same as for Fig. 5 except these samples were directly IP with anti-IκBα because they do not associate to RelA/p65 in these cell lines. A nonspecific (*ns*) band comes down in the IκBα IP that migrates directly above the IκBα band. The standard deviations for these samples were negligible and are not resolved at the given graphical resolution.

most likely caused by the tremendous stress that the cell experiences when its proteasomes are inactivated for prolonged periods of time. Consequently, no concrete conclusions could be made about the role of ubiquitination in the basal turnover of NF-κB-associated IκBα.

The signal-independent ubiquitination of free IκBα is quite robust (Fig. 7, *lanes 2–4*) which may explain the rapid basal turnover of free IκBα when compared that of NF-κB associated IκB (110 and 550 min, respectively). Free IκBα is very unstable in the absence of signal (Fig. 6*A*) and a detectable pool of ubiquitinated mutC and 3236mutC is observed in the absence of any stimulation and in the absence of a proteasome inhibitor (Fig. 7). The 3236 mutation in the mutC background does not reduce signal-independent ubiquitination of free IκBα (Fig. 7, *lane 4*). Therefore, phosphorylation of serine 32 and serine 36 is not necessary for efficient signal-independent ubiquitination and degradation of free IκBα.

We have previously shown that the carboxyl-terminal CKII sites are necessary for the efficient basal turnover of free IκBα (35, 46). Here we conclude (i) that efficient basal turnover of NF-κB-associated IκBα requires the carboxyl-terminal CKII phosphorylation and (ii) that IKK phosphorylation (serine 32 and 36) plays no role in the basal turnover of free or NF-κB associated IκBα.

DISCUSSION

IκBα functions as the primary regulator of NF-κB in both stimulated and unstimulated cells. To accomplish this, IκBα

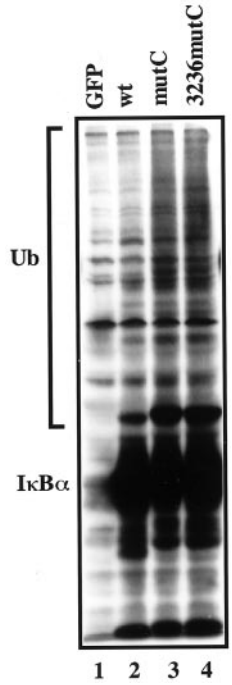

FIG. 7. **Signal-independent ubiquitination.** IκBα immunoblot of the given untreated stable IκBα −/− MEF pools in the absence of stimuli or proteasome inhibitor. *Ub,* ubiquitin.

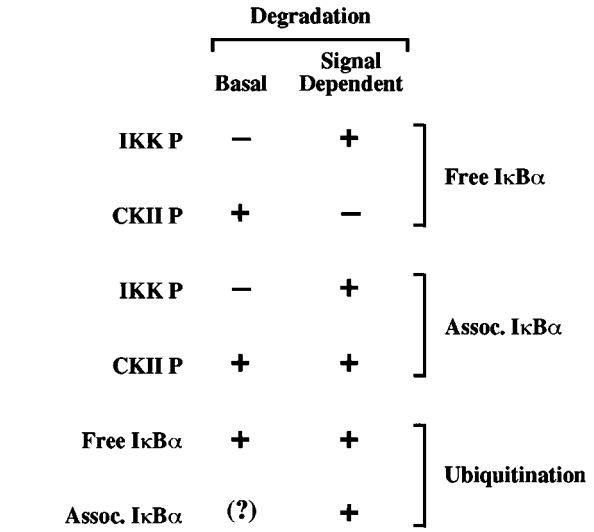

FIG. 8. **Role of phosphorylation and ubiquitination in IκBα degradation (summary).** The role of phosphorylation and ubiquitination in the regulation of signal-dependent and -independent degradation of free and NF-κB-associated IκBα. CKII phosphorylation (*CKII P*) is necessary for efficient basal degradation of both free and NF-κB-associated IκBα. Ubiquitination is involved in the basal turnover of free IκBα but it is still unclear whether or not ubiquitination plays a role in the basal turnover of NF-κB-associated IκBα. Signal-dependent degradation of free IκBα only requires IKK phosphorylation (*IKK P*) while efficient signal-dependent degradation of NF-κB-associated IκBα requires both CKII P and IKK P. Signal-dependent degradation of free and NF-κB-associated IκBα takes place in a ubiquitin-dependent manner.

itself must be a tightly regulated protein. NF-κB activity is positively and negatively regulated through IκBα phosphorylation and NF-κB-dependent synthesis of IκBα, respectively. Mechanisms that ensure proper NF-κB activity must exist to regulate signal-dependent degradation of free and NF-κB-associated IκBα as well as the signal-independent turnover of free and NF-κB-associated IκBα. These mechanisms also must allow newly synthesized IκBα the opportunity to enter the nucleus in order to remove NF-κB from the DNA and/or inhibit further activation upon the removal of signal (33). One way that the cell can easily and efficiently regulate the multiple states and fates of IκBα is through phosphorylation. Phosphorylation seems to be involved in almost all aspects of IκBαs regulation. We demonstrate when and how some of the different phosphorylation sites on IκBα can influence ubiquitination, degradation, and the overall stability of free and NF-κB-associated IκBα in the presence or absence of NF-κB inducing stimuli. Our conclusions about the role of phosphorylation and ubiquitination in the regulation of signal-dependent and -independent degradation of free and NF-κB-associated IκBα are summarized in Fig. 8.

Signal-dependent Degradation of NF-κB-associated IκBα—Several groups have shown that Ser-32 and Ser-36 undergo signal-dependent phosphorylation and that mutating these sites stabilizes IκBα in the presence of stimuli (18–21). There are many different views on the role of the carboxyl terminus and the CKII phosphorylation sites in signal-dependent degradation of IκBα (19, 21, 29, 35, 46–49). Using a variety of phosphorylation site mutations stably expressed in an IκBα −/− MEF cell line, we have confirmed that by mutating serine 32 and serine 36 to alanine, IκBα is stabilized in the presence of TNFα stimulation (18–21). We have also shown that the mutation of the carboxyl-terminal CKII sites (mutF) causes a significant stabilization of IκBα during TNFα stimulation but to a lesser extent than the 3236 mutation. Although work shown here was carried out using transduced IκBα −/− MEF cells, similar results were also obtained using transduced HeLa cell lines (data not shown). From these results we conclude that both IKK and CKII phosphorylation play an important role in the signal-dependent degradation of NF-κB-associated IκBα.

Experiments were also carried out to study the critical step of IκBα polyubiquitination during signal-dependent degradation of NF-κB-associated IκBα. *In vitro* and *in vivo* systems clearly showed that IκBα stability was increased in the 3236, M, and mutF mutants due to decreased levels of ubiquitination. The 3236 mutant simply blocks the signaling cascade from continuing to the ubiquitination step, while the mutF mutation is only able to decrease the efficiency of the reactions that lead to IκBα degradation. Thus, in agreement with others we conclude that both the amino-terminal IKK sites and the carboxyl-terminal CKII sites play significant roles in the signal-dependent degradation of NF-κB-associated IκBα. We also conclude that both amino-terminal IKK and carboxyl-terminal CKII phosphorylation is necessary for efficient signal-induced ubiquitination of NF-κB associated IκBα.

Signal-dependent Degradation of Free IκBα—To maintain a sensitive and rapidly responding NF-κB pathway the cell must be relatively free of unbound IκBα. If newly synthesized IκBα does not associate with NF-κB, the cell must clear this free population of IκBα before proper activation of NF-κB can take place. There is evidence that free IκBα is degraded in a signal-dependent manner and that this event is mainly regulated by the amino terminus of IκBα (45, 46). There are also reports that large carboxyl-terminal deletions that stretch into the sixth ankyrin repeat, and are believed to disrupt NF-κB association, can stabilize IκBα in the presence of stimuli (21, 47, 48, 50).

Here we investigated the role of the amino- and carboxyl-terminal phosphorylation sites in the context of a full-length IκBα molecule using both *in vitro* and *in vivo* systems. We were able to identify a mutant, mutC, that is degraded upon stimulation but does not associate to NF-κB. The mutC mutant contains five serine and threonine to alanine mutations in the ank6 region. The 3236 mutation in the mutC background is able to partially stabilize IκBα in the presence of TNFα without

having any effect on NF-κB DNA binding activity. The 3236mutC mutant also shows reduced signal-induced ubiquitination *in vivo* compared with the wild type levels seen in the presence of mutC alone (Fig. 4). A reduction in signal-dependent ubiquitination of free IκBα containing the 3236 mutation was also observed *in vitro*. Therefore the amino-terminal IKK phosphorylation sites play a critical role in signal-induced ubiquitination and degradation of free IκBα.

Several groups have reported on the effects of deleting or mutating the carboxyl-terminal PEST domain. Five groups report that the PEST domain does play a role in signal-dependent degradation (19, 21, 29, 45, 48), while four other groups report that it does not (46, 47, 49, 50). Here we examine free full-length IκBα containing five serine and threonine to alanine mutations in its PEST domain (mutF). In our *in vitro* ubiquitination system the mutF mutation caused a slight increase in the extent of signal-induced ubiquitination of free IκBα when compared with wild type IκBα. This is in contrast to the decrease in ubiquitination that was seen for mutF while in association with NF-κB. In the presence of NF-κB inducing stimuli, the differential regulation of IκBα stability by the carboxyl-terminal CKII sites indicates that a very interesting and sophisticated mechanism is utilized to regulate the different populations of IκBα during signaling. We conclude that CKII phosphorylation is necessary for efficient signal-dependent degradation of NF-κB-associated IκBα, but it is not necessary for efficient signal-dependent degradation of free IκBα (46). In fact, the absence of CKII phosphorylation on free IκBα, under stimulated conditions, may potentiate the rate at which it is ubiquitinated and subsequently degraded by the 26 S proteasome. This ensures that NF-κB activation is maintained until stimuli is removed and IKK activation is down-regulated.

Signal-independent Turnover of NF-κB-associated IκBα—IκBα is a more stable molecule while associated to NF-κB than in its free state (45, 51, 52). However, in the associated state, IκBα is not totally stabilized because NF-κB activation and IκBα degradation can be observed within a couple of hours after treating cells with cyclohexamide to block new protein synthesis (53, 54). This activation is due to the signal-independent turnover of the NF-κB-associated IκBα. We show that mutating the IKK phosphorylation sites in IκBα has no effect on the basal degradation rate of NF-κB-associated IκBα (Fig. 5, *A* and *C*). On the other hand, mutating the CKII sites caused IκBαs half-life to double (Fig. 5*B*). We were unable to determine if ubiquitination plays a significant role in the basal turnover of NF-κB-associated IκBα because long term treatment (2–4 h) of the cells with β-lactone seems to activate the NF-κB pathway. A role for ubiquitination in the basal turnover of NF-κB-associated IκBα may be observed in Fig. 7 (comparing *lanes 1* and *2*). It appears that there may be a detectable pool of ubiquitinated IκBα even in the absence of an NF-κB inducing signal. Therefore, we conclude that only the constitutive CKII phosphorylation sites are important for signal-independent degradation of NF-κB-associated IκBα.

Basal Turnover of Free IκBα—We have previously shown that both a carboxyl-terminal deletion of 39 amino acids and that the mutF mutation can increase the stability of free IκBα in the absence of signal (35, 46, 49). In this report we go on to show that free IκBα is very efficiently ubiquitinated in the absence of signal, and that polyubiquitinated IκBα can be detected even in the absence of a proteasome inhibitor (Fig. 7, *lane 3*). We also demonstrate that the 3236 mutation is unable to block ubiquitination of free IκBα in the absence of signal (Fig. 7, *lane 4*). We propose that only the CKII sites are significant regulators of the signal-independent degradation of free IκBα, and that this degradation is mediated by ubiquitination and the 26 S proteasome.

An important component of IκBα regulation/degradation that still needs to be identified is the signal-independent ubiquitin conjugation-ligase complex. We have shown that free and possibly NF-κB-bound IκBα undergo signal-independent ubiquitination. It has also been shown that the IκB-ligase complex, that was recently identified (22, 23, 55, 56), only binds to and ubiquitinates IκBα when it is phosphorylated at its signal-induced phosphorylation sites (22, 23, 55–57). This suggests that a signal-independent IκBα ubiquitin-ligase complex must exist. It is possible that this signal-independent ubiquitin ligase recognizes the same residues as the signal-dependent ligase but only when serine 32 and serine 36 are unphosphorylated. When IκBα is bound to NF-κB the signal-independent ligase may only be able to associate to IκBα with a weak affinity due to steric hindrance caused by IκBαs association to NF-κB. When IκBα is free in the cell the steric hindrance may be relieved allowing the signal-independent ubiquitin ligase to bind to IκBα with a much higher affinity. This would explain the differences in the rate of ubiquitination and basal turnover between free and NF-κB-bound IκBα. This could also explain why we observed an increase in the basal turnover of free IκBα containing the 3236 mutation (Fig. 6*B*). By changing serine 32 and serine 36 to alanine we may have created a molecule with an even higher affinity for the signal-independent ubiquitin-ligase complex. An increase in the affinity of this ligase for its target would most likely result in an increase in the rate of its ubiquitination and subsequent degradation. Identification of the signal-independent ubiquitin ligase would allow further dissection of the mechanisms involved in IκBα regulation.

Although our results are internally consistent and supported by previous biochemical and structural studies it is always necessary to consider the possibility that introducing mutations into any given protein may have unintended effects. It is for this reason that we utilized point mutations rather than gross deletions as has been done in other studies. It was our hope that by minimizing the changes made to IκBα and by utilizing full-length proteins that we would be able to obtain the most meaningful results.

In summary, we have investigated the role of phosphorylation in the regulation of IκBα stability. We have demonstrated that specific phosphorylation events can have very different implications depending on the state of the cell and of IκBα. The complexity of the regulation of IκBα is a reflection of its importance to the cell. It will be interesting and of much importance to further explore the effects that other components of the IκB kinase complex and degradation machinery have on the phosphorylation, ubiquitnation, and degradation of IκBα.

Acknowledgments—We thank C. L. Stewart for providing the IκBα (−/−) cells. We thank S. Tan for support and critical reading of the manuscript, B. Coyne for secretarial assistance, and all members of the Verma laboratory for helpful suggestions and scientific discussions.

REFERENCES

1. Baeuerle, P. A., and Baltimore, D. (1996) *Cell* **87,** 13–20
2. Verma, I. M., Stevenson, J. K., Schwarz, E. M., Van Antwerp, D., and Miyamoto, S. (1995) *Genes Dev.* **9,** 2723–2735
3. Miyamoto, S., and Verma, I. M. (1995) *Adv. Cancer Res.* **66,** 255–292
4. Baeuerle, P. A., and Henkel, T. (1994) *Annu. Rev. Immunol.* **12,** 141–179
5. Siebenlist, U., Franzoso, G., and Brown, K. (1994) *Annu. Rev. Cell Biol.* **10,** 405–455
6. Finco, T. S., and Baldwin, A. S. (1995) *Immunity* **3,** 263–272
7. Thanos, D., and Maniatis, T. (1995) *Cell* **80,** 529–532
8. Wulczyn, F. G., Krappmann, D., and Scheidereit, C. (1996) *J. Mol. Med.* **74,** 749–769
9. Baltimore, D., and Beg, A. A. (1995) *Nature* **373,** 287–288
10. Beg, A. A., and Baldwin, A. S., Jr. (1993) *Genes Dev.* **7,** 2064–2070
11. Jacobs, M. D., and Harrison, S. C. (1998) *Cell* **95,** 749–758
12. Huxford, T., Huang, D. B., Malek, S., and Ghosh, G. (1998) *Cell* **95,** 759–770
13. DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. (1997) *Nature* **388,** 548–554
14. Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J.,

Young, D. B., Barbosa, M., Mann, M., Manning, A., and Rao, A. (1997) *Science* **278,** 860–866
15. Regnier, C. H., Song, H. Y., Gao, X., Goeddel, D. V., Zhaodan, C., and Rothe, M. (1997) *Cell* **90,** 373–383
16. Woronicz, J. D., Gao, X., Cao, Z., Rothe, M., and Goeddel, D. V. (1997) *Science* **278,** 866–869
17. Zandi, E., Rothwarf, D. M., Delhase, M., Hayakawa, M., and Karin, M. (1997) *Cell* **91,** 243–252
18. Brockman, J. A., Scherer, D. C., McKinsey, T. A., Hall, S. M., Qi, X., Lee, W. Y., and Ballard, D. W. (1995) *Mol. Cell. Biol.* **15,** 2809–2818
19. Brown, K., Gerstberger, S., Carlson, L., Franzoso, G., and Siebenlist, U. (1995) *Science* **267,** 1485–1488
20. Traenckner, E. B., Pahl, H. L., Henkel, T., Schmidt, K. N., Wilk, S., and Baeuerle, P. A. (1995) *EMBO J.* **14,** 2876–2883
21. Whiteside, S. T., Ernst, M. K., LeBail, O., Laurent-Winter, C., Rice, N., and Israeel, A. (1995) *Mol. Cell. Biol.* **15,** 5339–5345
22. Yaron, A., Hatzubai, A., Davis, M., Lavon, I., Amit, S., Manning, A. M., Andersen, J. S., Mann, M., Mercurio, F., and Ben-Neriah, Y. (1998) *Nature* **396,** 590–594
23. Spencer, E., Jiang, J., and Chen, Z. J. (1999) *Genes Dev.* **13,** 284–294
24. Scherer, D. C., Brockman, J. A., Chen, Z., Maniatis, T., and Ballard, D. W. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 11259–11263
25. Rodriguez, M. S., Wright, J., Thompson, J., Thomas, D., Baleux, F., Virelizier, J. L., Hay, R. T., and Arenzana-Seisdedos, F. (1996) *Oncogene* **12,** 2425–2435
26. Baldi, L., Brown, K., Franzoso, G., and Siebenlist, U. (1996) *J. Biol. Chem.* **271,** 376–379
27. Palombella, V. J., Rando, O. J., Goldberg, A. L., and Maniatis, T. (1994) *Cell* **78,** 773–785
28. Traenckner, E. B., and Baeuerle, P. A. (1995) *J. Cell Sci. Suppl.* **19,** 79–84
29. Chen, Z., Hagler, J., Palombella, V. J., Melandri, F., Scherer, D., Ballard, D., and Maniatis, T. (1995) *Genes Dev.* **9,** 1586–1597
30. Chiao, P. J., Miyamoto, S., and Verma, I. M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 28–32
31. Liou, H. C., and Baltimore, D. (1993) *Curr. Opin. Cell Biol.* **5,** 477–487
32. Beg, A. A., Ruben, S. M., Scheinman, R. I., Haskill, S., Rosen, C. A., and Baldwin, A. S., Jr. (1992) *Genes Dev.* **6,** 1899–1913
33. Arenzana-Seisdedos, F., Thompson, J., Rodriguez, M. S., Bachelerie, F., Thomas, D., and Hay, R. T. (1995) *Mol. Cell. Biol.* **15,** 2689–2696
34. Barroga, C. F., Stevenson, J. K., Schwarz, E. M., and Verma, I. M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 7637–7641
35. Schwarz, E. M., Van Antwerp, D., and Verma, I. M. (1996) *Mol. Cell. Biol.* **16,** 3554–3559
36. Shirakawa, F., and Mizel, S. B. (1989) *Mol. Cell. Biol.* **9,** 2424–2430
37. Haskill, S., Beg, A. A., Tompkins, S. M., Morris, J. S., Yurochko, A. D., Sampson-Johannes, A., Mondal, K., Ralph, P., and Baldwin, A. S., Jr. (1991) *Cell* **65,** 1281–1289
38. Imbert, V., Rupec, R. A., Livolsi, A., Pahl, H. L., Traenckner, E. B., Mueller-Dieckmann, C., Farahifar, D., Rossi, B., Auberger, P., Baeuerle, P. A., and Peyron, J. F. (1996) *Cell* **86,** 787–798
39. Klement, J. F., Rice, N. R., Car, B. D., Abbondanzo, S. J., Powers, G. D., Bhatt, P. H., Chen, C. H., Rosen, C. A., and Stewart, C. L. (1996) *Mol. Cell. Biol.* **16,** 2341–2349
40. Umesono, K., Murakami, K. K., Thompson, C. C., and Evans, R. M. (1991) *Cell* **65,** 1255–1266
41. Morgenstern, J. P., and Land, H. (1990) *Nucleic Acids Res.* **18,** 3587–3596
42. Wigler, M., Pellicer, A., Silverstein, S., and Axel, R. (1978) *Cell* **14,** 725–731
43. Andrews, N. C., and Faller, D. V. (1991) *Nucleic Acids Res.* **19,** 2499
44. Inoue, J., Kerr, L. D., Ransone, L. J., Bengal, E., Hunter, T., and Verma, I. M. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 3715–3719
45. Krappmann, D., Wulczyn, F. G., and Scheidereit, C. (1996) *EMBO J.* **15,** 6716–6726
46. Van Antwerp, D. J., and Verma, I. M. (1996) *Mol. Cell. Biol.* **16,** 6037–6045
47. Aoki, T., Sano, Y., Yamamoto, T., and Inoue, J. I. (1996) *Oncogene* **12,** 1159–1164
48. Rodriguez, M. S., Michalopoulos, I., Arenzana-Seisdedos, F., and Hay, R. T. (1995) *Mol. Cell. Biol.* **15,** 2413–2419
49. Lin, R., Beauparlant, P., Makris, C., Meloche, S., and Hiscott, J. (1996) *Mol. Cell. Biol.* **16,** 1401–1409
50. Sun, S., Elwood, J., and Greene, W. C. (1996) *Mol. Cell. Biol.* **16,** 1058–1065
51. Rice, N. R., and Ernst, M. K. (1993) *EMBO J.* **12,** 4685–4695
52. Scott, M. L., Fujita, T., Liou, H. C., Nolan, G. P., and Baltimore, D. (1993) *Genes Dev.* **7,** 1266–1276
53. Sen, R., and Baltimore, D. (1986) *Cell* **46,** 705–716
54. Baeuerle, P. A., and Baltimore, D. (1988) *Science* **242,** 540–546
55. Kroll, M., Margottin, F., Kohl, A., Renard, P., Durand, H., Concordet, J. P., Bachelerie, F., Arenzana-Seisdedos, F., and Benarous, R. (1999) *J. Biol. Chem.* **274,** 7941–7945
56. Suzuki, H., Chiba, T., Kobayashi, M., Takeuchi, M., Furuichi, K., and Tanaka, K. (1999) *Biochem. Biophys. Res. Commun.* **256,** 121–126
57. Maniatis, T. (1999) *Genes Dev.* **13,** 505–510