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N- and C-Terminal Sequences Control Degradation of MAD3/IκBα in Response to Inducers of NF-κB Activity

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The proteolytic degradation of the inhibitory protein MAD3/IκBα in response to extracellular stimulation is a prerequisite step in the activation of the transcription factor NF-κB. Analysis of the expression of human IκBα protein in stable transfectants of mouse 70Z/3 cells shows that, as for the endogenous murine protein, exogenous IκBα is degraded in response to inducers of NF-κB activity, such as phorbol myristate acetate or lipopolysaccharide. In addition, pretreatment of the cells with the proteasome inhibitor N-Ac-Leu-Leu-norleucinal inhibits this ligand-induced degradation and, in agreement with previous studies, stabilizes a hyperphosphorylated form of the human IκBα protein. By expressing mutant forms of the human protein in this cell line, we have been able to delineate the sequences responsible for both the ligand-induced phosphorylation and the degradation of IκBα. Our results show that deletion of the C terminus of the IκBα molecule up to amino acid 279 abolishes constitutive but not ligand-inducible phosphorylation and inhibits ligand-inducible degradation. Further analysis reveals that the inducible phosphorylation of IκBα maps to two serines in the N terminus of the protein (residues 32 and 36) and that the mutation of either residue is sufficient to abolish ligand-induced degradation, whereas both residues must be mutated to abolish inducible phosphorylation of the protein. We propose that treatment of 70Z/3 cells with either phorbol myristate acetate or lipopolysaccharide induces a kinase activity which phosphorylates serines 32 and 36 and that these phosphorylations target the protein for rapid proteolytic degradation, possibly by the ubiquitin-26S proteasome pathway, thus allowing NF-κB to translocate to the nucleus and to activate gene expression.

The Rel/NF-κB family of proteins (p50, p52, p65/RelA, c-Rel, and RelB) bind specifically as homo- or heterodimers to κB motifs located in the promoters and enhancers of a large number of genes that control various aspects of the immune and inflammatory responses (including the major histocompatibility complex class I genes, the immunoglobulin κ light chain, interleukin 2 and its receptor, interleukins 6 and 8, granulocyte-macrophage colony-stimulating factor, beta interferon, and T-cell receptor β chain) as well as those of several viruses, including human immunodeficiency virus types 1 and 2 and cytomegalovirus (for recent reviews, see references 2 and 32). In most cell types, NF-κB is maintained in an inactive, cytoplasmic state in complexes with members of the IκB family, which includes IκBα, IκBβ, and IκBγ (only B cells and some cells of the monocyte or macrophage lineage exhibit constitutive nuclear NF-κB activity). These inhibitors all contain multiple copies of a motif, the ankyrin repeat, which interact with and inhibit the nuclear localization and DNA binding of the Rel/NF-κB proteins (for a review, see reference 3). NF-κB is induced by a wide variety of stimuli (including phorbol myristate acetate [PMA], tumor necrosis factor, interleukin 1, lipopolysaccharide [LPS], viral infection, and many mitogens and cytokines) through a mechanism involving inactivation of IκBα by proteolytic degradation, thus enabling free NF-κB to translocate to the nucleus and to activate the transcription of its target genes (4, 7, 8, 11, 20, 26, 31, 33). Pretreatment of cells with proteasome inhibitors has shown that IκBα becomes phosphorylated in response to a variety of stimuli, although this phosphorylation is not sufficient, per se, to dissociate the inhibitor from the NF-κB complex. It is believed that this phosphorylated form of IκBα is the substrate for proteolytic degradation, which probably occurs in the context of the NF-κB/IκB complex (1, 9, 18, 21, 23, 36).

The domains of IκBα responsible for the interaction with Rel proteins as well as for inhibition of their binding to DNA have been well characterized (see reference 10 and references therein). To establish the domains of the IκBα protein responsible for degradation and to map the sites of ligand-induced phosphorylation, we have taken advantage of the fact that in mouse 70Z/3 cells, human IκBα expressed from a stably transfected construct is degraded in response to extracellular stimuli. Pretreatment of the cells with the proteasome inhibitor N-Ac-Leu-Leu-norleucinal (ALLN) blocks this induced degradation and stabilizes a hyperphosphorylated form of the transfected IκBα protein. Mutagenesis of the human IκBα reveals that sequences C terminal of the ankyrin repeats contain sites of constitutive phosphorylation, while inducible hyperphosphorylation takes place on serines 32 and 36. The phosphorylation of both of these residues is apparently required for the transfected IκBα protein to be degraded in response to extracellular stimuli such as PMA or LPS, a process which is dependent upon the C-terminal PEST sequences (sequences rich in P, E, D, S, and T) of IκBα.

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MATERIALS AND METHODS

Cells. HeLa cells were grown in Dulbecco minimal essential medium (DMEM) supplemented with 10% newborn calf serum. 70Z/3, a murine pre-B cell line, was maintained in RPMI medium supplemented with 10% fetal calf serum and 50 μM β-mercaptoethanol.

Antisera. The antisera used were the following (see also references 26 and 27). Anti-c-Rel antisera were 1050, raised against amino acids 438 to 455 of the murine protein, and 1051, raised against amino acids 498 to 513 of murine c-Rel. Anti-RelA antisera were 1207 (RelAN), raised against amino acids 2 to 18 of the human protein, and 1226 (RelAC), raised against amino acids 537 to 550 of human RelA. Anti-IκBα antisera were 1309 (anti-IκBαN) and 296 (anti-IκBαC), raised against amino acids 2 to 17 and 310 to 317 of human IκBα respectively. Other antisera were 751 (anti-murine IκBα), raised against amino acids 2 to 15 of murine IκBα; 32 (anti-rat IκBα), raised against amino acids 48 to 65 of rat IκBα; and 48068, raised against recombinant human IκBα. Serum 1309 recognizes human IκBα much better than it does mouse IκBα, and the reverse is true for serum 751. The S7 antiserum (anti-IκBα), a gift of R. T. Hay (St. Andrews, United Kingdom), was raised against recombinant human IκBα.

Plasmids. Expression vectors for transfection into 70Z/3 cells were obtained by subcloning cDNAs encoding IκBα or its derivatives into the plasmid pRc-CMV (InVitrogen). Some of the mutants were first cloned into the plasmid pT7βplink (24), and then the fragment containing the β-globin 5′ untranslated region (UTR) and IκBα encoding sequences was recloned into pRc-CMV. Deletions of IκBα were produced either by utilization of restriction sites or by digestion with *Exo*III. Mutations of IκBα were constructed by site-directed mutagenesis with PCR or with the Bio-Rad Muta-Gene Phagemid In Vitro Mutagenesis system, version 2 (the sequence of the oligonucleotides is available from us upon request). Both types of fragment were cloned into either pRc-CMV or pCDNA3 (InVitrogen). All constructs were linearized with *Bgl*II before transfection by electroporation into 70Z/3 cells.

Cytoplasmic extracts. To prepare crude cytoplasmic extracts, 5×10^6 cells were washed twice with phosphate-buffered saline and then lysed in 50 μl of Dignam buffer A supplemented with 5 mM NaF, 2.5 mM Na_3VO_4, 2.5 mM orthophosphate, and 0.2% Nonidet P-40. After centrifugation for 2 min in a microcentrifuge, the supernatant was transferred to a clean tube for further analysis. Protein concentrations were determined by a Bradford assay (Bio-Rad).

Transfections. 70Z/3 cells were grown to a density of 10^6 cells per ml, pelleted, and resuspended in complete medium at 5×10^6 cells per 0.5 ml, and then the cells were electroporated in 4-mm cuvettes with a Eurogentec Celljject eletroporator at 260 V, 1500 μF, and infinite resistance. Cells were diluted into 4 ml of complete medium and were left to recover overnight before addition of 0.7 mg of G418 per ml. Once established, clones were maintained in complete medium supplemented with 0.3 mg of G418 per ml.

Two-dimensional (2D) gel electrophoresis. Cytoplasmic extracts from 25×10^6 cells were prepared, and 600 μg of protein was made up to 30 μl of Dignam buffer A supplemented with 1% β-mercaptoethanol and 0.3% sodium dodecyl sulfate (SDS). Samples were boiled for 3 min and then flash-frozen in liquid nitrogen. After lyophilization, samples were resuspended in 30 μl of sample buffer (9.95 M urea, 4% Nonidet P-40, 2% ampholyte [pH 5 to 7], and 100 mM dithiothreitol) and centrifuged for 2 min. Samples were then loaded onto the isoelectrofocusing gel (pH range, 4 to 8; Millipore Corporation) and run for 20,000 V · h. The second dimension was performed as previously described on a 12.5% acrylamide gel (15). Relative isoelectric points were determined by parallel migration of a carbamylated muscle creatine phosphokinase standard (BDH), and the relative molecular masses of the proteins were determined according to molecular weight markers applied to an adjacent slot on the same gel. After SDS-polyacrylamide gel electrophoresis (PAGE), proteins were blotted as they were for one-dimensional polyacrylamide gels.

Immunoblots. Immunoblots were incubated with anti-IκBα sera diluted 1:1,000, as indicated in the figure legends, and proteins were revealed with the Amersham ECL system.

Immunoprecipitations. 70Z/3 cells were metabolically labelled with either L-[^{35}S]methionine–L-[^{35}S]cysteine or $^{32}P_i$ and were lysed, and immune complexes were precipitated as described in the figure legends. Cells from the cell line 293 were metabolically labelled and immune complexes were precipitated as previously described (10).

Thrombin treatment. Thrombin treatment of immune complexes was carried out as described by MacKichan et al. (18a). Cytoplasmic extracts of 70Z/3 clones, either untreated or stimulated with 10 μg of LPS per ml for 45 min in the presence of 100 μM ALLN, were boiled for 5 min in 1% SDS, and this was followed by immunoprecipitation with antiserum 296 directed against the C terminus of IκBα. The protein A-Sepharose beads carrying the immune complexes were washed three times in radioimmunoprecipitation assay buffer without SDS and then were subjected to three washes in thrombin buffer (20 mM Tris-HCl, pH 8.3, 150 mM NaCl, 2.5 mM $CaCl_2$, and 10% glycerol). After resuspension in 20 μl of thrombin buffer containing 3 U of thrombin, digestion was carried out for 2 to 16 h at room temperature. All buffers contained phosphatase inhibitors. After centrifugation, the supernatant was loaded on a 16.5% Tricine gel (29) which was then transferred onto an Immobilon membrane (Millipore). The membrane was blotted with antiserum 1309, directed against the N terminus of IκBα.

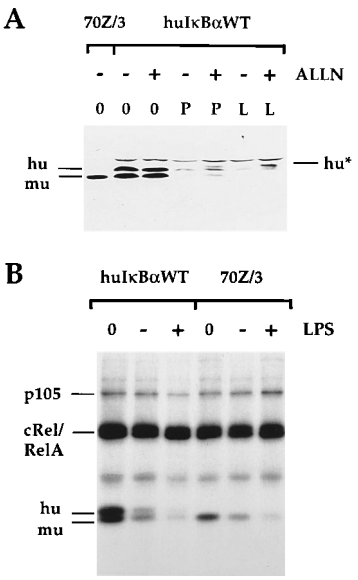

FIG. 1. (A) 70Z/3 cells transfected with vector plasmid alone (70Z/3) or cells expressing human IκBα (huIκBαWT) were grown to a density of 10^6 cells per ml and then were pelleted, resuspended at 5×10^6 cells per ml, and incubated for 45 min in the presence (+) or absence (−) of the proteasome inhibitor ALLN (100 μM). Cells were then treated with either 50 ng of PMA (P) per ml for 10 min or 10 μg of LPS (L) per ml for 45 min or were left untreated (0), and cytoplasmic extracts were prepared and analyzed by Western blotting with anti-IκBα antiserum S7. The positions of the murine (mu), human (hu), and more slowly migrating hyperphosphorylated human (hu*) proteins are indicated. (B) 70Z/3 cells transfected with vector plasmid alone (70Z/3) or cells expressing human IκBα (huIκBαWT) were labelled for 1 h with 100 μCi of L-[^{35}S]cysteine–L-[^{35}S]methionine per ml and then were diluted into prewarmed, complete medium containing 50 mM L-cysteine–L-methionine. One third of the cells were lysed immediately (0; see below). The remaining cells were either treated with LPS (10 μg/ml) for 90 min (+) or left untreated (−) before being lysed in TNT (20 mM Tris, pH 7.5, 250 mM NaCl, 1% Triton X-100) and centrifuged at 10,000 × *g* for 10 min, and then the supernatants were precipitated with anti-Rel (1050) and anti-RelA (1226) antisera. After being washed, proteins were eluted with the corresponding peptides and then were reprecipitated with anti-Rel (1051) and anti-RelA (1207) antisera. The positions of p105, c-Rel, RelA, and human (hu) and murine (mu) IκBα proteins are indicated to the left of the figure.

RESULTS

A stably transfected IκBα protein is degraded in response to extracellular stimuli. In order to identify the sequences of the IκBα protein responsible for its degradation in response to extracellular stimuli, we chose to utilize a system in which mouse 70Z/3 cells were stably transfected with plasmids expressing human IκBα protein. Endogenous murine IκBα and the human IκBα could be distinguished by their different mobilities after SDS-PAGE (compare the first two lanes of Fig. 1A). The promoter chosen (the cytomegalovirus promoter) has a weak activity in 70Z/3 cells, and thus the levels of transfected protein do not differ greatly from those seen for the endogenous protein (Fig. 1B). A cell line expressing the wild-type (WT) human IκBα protein was treated with either PMA or LPS, and the two IκBα proteins were analyzed by Western blotting (immunoblotting). As can be seen in Fig. 1A, both the human and mouse IκBα proteins are degraded in response to these stimuli. In addition, immunoprecipitation with anti-RelA and anti-c-Rel antibodies demonstrates that the transfected IκBα is associated with the Rel proteins to an extent similar to that of the endogenous murine protein and that IκBα undergoes degradation with kinetics similar to those of the endogenous murine protein (Fig. 1B). Immunodepletions with a mixture of antisera directed against p50, RelA, and c-Rel resulted

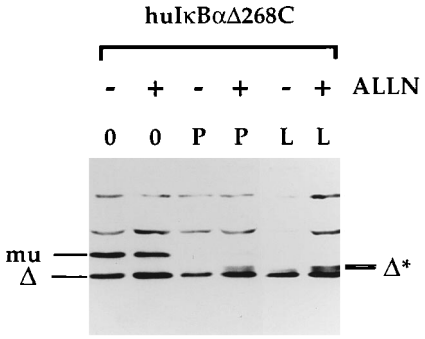

FIG. 2. Cells transfected with the Δ268C mutant were prepared as described in the legend to Fig. 1A and then were incubated for 45 min in the presence (+) or absence (−) of ALLN. After incubation with 50 ng of PMA (P) per ml for 10 min or 10 μg of LPS (L) per ml for 45 min, cytoplasmic extracts were prepared and IκBα proteins were analyzed by immunoblotting. The positions of the endogenous murine (mu), transfected human Δ268C (Δ), and electrophoretically retarded human Δ268C (Δ*) proteins are indicated. The Δ* band sometimes appears as a doublet, but this result has not been found consistently.

in about 10% of transfected WT IκBα being left in the supernatant (compared with the total amount of IκBα left in the supernatant when a preimmune serum was used; data not shown). The result was the same for the endogenous murine IκBα, indicating that the levels of free IκBα are comparable for both transfected and endogenous proteins. In accordance with results obtained by others (1, 9, 18, 21, 23, 36), we observed a stabilization of both murine and human proteins after pretreatment of the cells with the proteasome inhibitor ALLN (Fig. 1A, lanes 5 and 7 compared with lanes 4 and 6, respectively); the murine protein is somehow less sensitive to the effect of ALLN, as previously observed by others (see Fig. 2A of reference 6). Human IκBα also exhibits a characteristic upshift in its electrophoretic mobility, presumably resulting from the stabilization of a hyperphosphorylated form of the IκBα molecule after pretreatment with ALLN. Under these conditions, no change in the electrophoretic mobility of the murine IκBα protein was seen (Fig. 1A and data not shown), although this protein is normally degraded following stimulation. In addition, 2D gel analysis shows a shift of the isoforms of the murine protein towards the acidic region following stimulation (data not shown), thus confirming the fact that murine IκBα is also hyperphosphorylated under these conditions. Treatment of resting cells with ALLN has no effect upon the mobility of either protein during SDS-PAGE (Fig. 1A, compare lanes 2 and 3). Using this approach, we have stably transfected various mutant human IκBα constructs in order to localize the sequences in the human protein responsible for its inducible phosphorylation and degradation.

Deletion of IκBα PEST sequences abolishes degradation and constitutive, but not ligand-induced, phosphorylation. The carboxy terminus of IκBα contains PEST sequences, which have been suggested to function as instability determinants for proteins degraded by the ubiquitin-26S proteasome pathway (25). Since IκBα degradation has been shown to be sensitive to inhibitors of proteasome activity, we first investigated whether this region of the protein was important for the inducible phosphorylation and degradation of the IκBα protein. We constructed a series of C-terminal deletion mutants and stably transfected them into 70Z/3 cells. As shown in Fig. 2, deletion up to and including amino acid 269 (mutant Δ268C), which eliminates most of the PEST sequences, abolished the degradation of the transfected protein in response to PMA or LPS. Similar results have been obtained in pulse-chase experiments (data not shown). The truncated protein is still able to interact with NF-κB in vivo, as judged by its ability to retain cotransfected RelA in the cytoplasm of 293 or COS cells (data not shown). In extracts pretreated with ALLN before PMA or LPS stimulation, the transfected protein was subject to a retardation in mobility (Fig. 2, lanes 4 and 6), indicating that it could still be inducibly phosphorylated in response to external signalling. Longer exposure of the filter reveals that this upshifted band is also present when cells are induced in the absence of ALLN (data not shown). The mutation of Y-251 to an aspartic acid, or the mutation of S-252, S-262, or T-263 to an alanine, did not affect either phosphorylation or degradation in response to induction with PMA or LPS (data not shown; these are phosphorylatable residues located between the end of the ankyrin repeats and amino acid 268), indicating that phosphorylation sites lie elsewhere in the molecule. The deletion of sequences up to and including amino acid 279 (Δ278C) also stabilizes the protein against ligand-induced degradation, while a protein deleted up to amino acid 291 (Δ290C) behaves as the WT protein (data not shown), indicating that amino acids 279 to 290 play an important role in induced degradation of IκBα.

While both human and murine full-length proteins exist as multiple isoforms in uninduced cells as revealed by multiple spots following 2D gel electrophoresis and immunoblotting (Fig. 3A), deletion of the C terminus of IκBα (mutant Δ268C) results in the appearance of a single spot (Fig. 3B), indicating that the C-terminal PEST sequences are variably phosphorylated in uninduced cells. To more directly demonstrate that constitutive phosphorylation takes place in the PEST region of the molecule, we carried out immunoprecipitation on $^{32}P_i$-labelled cells transfected either with the WT human IκBα or with the mutant Δ268C. The results shown in Fig. 4 demonstrate that WT human IκBα is already phosphorylated in unstimulated cells (Fig. 4B, lane 3) and that treatment with LPS in the presence of ALLN results in the appearance of an additional band with reduced mobility (lane 4) (^{32}P labelling results in a poor resolution of the two bands, as opposed to the results obtained by ECL on Western blots); on the other hand, a very faint band can be detected in the case of the Δ268C mutant in unstimulated cells (lane 1; clearly visible on a longer exposure), while LPS treatment results in the appearance of an electrophoretically retarded labelled band. Western blotting of controls of these extracts shows the presence of the WT and Δ268C proteins in unstimulated cells as well as the upshifting in the presence of LPS and ALLN (Fig. 4A). These results demonstrate that almost all basal phosphorylation takes place between amino acids 269 and 317 and that inducible phosphorylation takes place in the remaining part of the molecule. Since the mutations of serine and threonine residues to alanines in the PEST region have only slight effects upon the ability of IκBα to inhibit DNA binding of NF-κB (10) and have no effect upon the degradation of IκBα in response to stimulation (data not shown), the function of these constitutive phosphorylations of IκBα remains, for the moment, unclear (see Discussion).

Phosphorylation of both serines 32 and 36 is required for ligand-induced degradation of transfected IκBα. As deletion of the C-terminal region of IκBα had no effect upon its ability to be phosphorylated in response to extracellular stimuli, we next investigated phosphorylatable residues in the N terminus of the molecule. Deletion of the first 30 amino acids of IκBα (Δ30N) destabilized the protein to such an extent that it was not visible by either Western blotting or immunoprecipitation of [^{35}S]methionine-labelled extracts from cells stably transfected with this construct (data not shown).

Analysis of IκBα sequences (Fig. 5A) reveals that the N

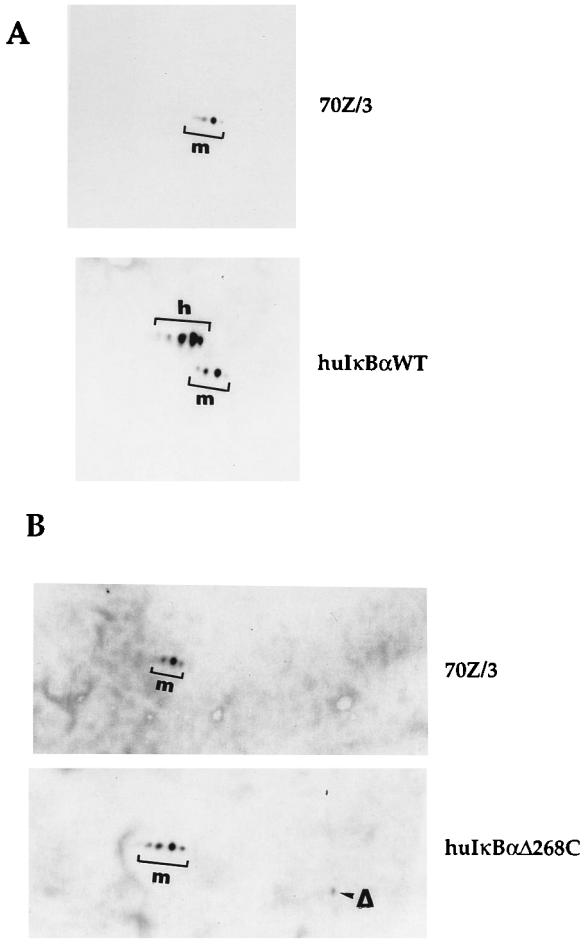

FIG. 3. Proteins were analyzed by 2D gel electrophoresis as described in Materials and Methods. The positions of the murine (m), WT human (h), and truncated human (Δ) IκBα proteins are indicated. The acidic side of the gel is on the left. This experiment has been repeated several times with identical results. (A) 70Z/3, 70Z/3 cells transfected with pRc-CMV; huIκBαWT, 70Z/3 cells expressing WT human IκBα. (B) huIκBαΔ268C, 70Z/3 cells expressing human IκBαΔ268C.

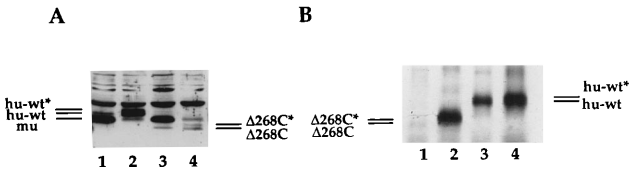

FIG. 4. 70Z/3 cells transfected with WT human IκBα or with the Δ268C mutant were starved for 1 h in phosphate-free medium supplemented with 3% dialyzed calf serum and then were labelled for 3 h with 0.5 mCi of $^{32}P_i$ per ml in the presence of 100 μM ALLN. Half of the cells were stimulated for 45 min with LPS (10 μg/ml; lanes 2 and 4) at the end of the labelling period, while the other half were left untreated (lanes 1 and 3). Cytoplasmic extracts of the cells were then prepared in the presence of phosphatase inhibitors. (A) Aliquots of the stimulated or unstimulated cells were left unlabelled, and cytoplasmic extracts were analyzed by Western blotting with the S7 antiserum raised against recombinant human IκBα (essentially identical results were obtained by subjecting the labelled extracts to precipitation with antiserum 1309 followed by immunoblotting). Lanes 1 and 2, human WT IκBα; lanes 3 and 4, Δ268C. mu, endogenous murine IκBα. (B) ^{32}P-labelled cytoplasmic extracts were immunoprecipitated with antiserum 48068 raised against recombinant human IκBα (the amounts of extract were adjusted to contain approximately identical amounts of WT or Δ268C proteins, as determined from the immunoblot shown in panel A). Following boiling in 1% SDS, a second immunoprecipitation was carried out, with serum 1309 (which does not recognize the murine molecule) raised against the N terminus of human IκBα, and then extracts were subjected to analysis on a 12% SDS gel. Lanes 1 and 2, Δ268C; lanes 3 and 4, WT human IκBα. Asterisks indicate the hyperphosphorylated forms of the given molecules.

terminus of the molecule contains two highly conserved serine or threonine residues (within the IκBα or IκBβ family). We therefore decided to mutate these residues and to analyze the behavior of the mutant proteins in response to PMA and LPS when stably expressed in 70Z/3 cells (residue 62, which in the human protein is also a serine, is not conserved in murine IκBα and therefore has not been investigated further; mutation of threonine at position 71 to an alanine results in a molecule with a greatly reduced affinity for the Rel proteins and a very short half-life, thus precluding its analysis). The mutation of either serine 32 (Fig. 5B and D) or serine 36 (Fig. 5C) to an alanine abolished the degradation of the transfected protein following stimulation with PMA or LPS. Since the mutation of serine 36 to alanine resulted in a protein (S36A) with an altered electrophoretic migration, we also tested a mutant in which serine 36 was changed to a proline. This protein was also not degraded in response to PMA (Fig. 5B) or LPS (Fig. 5D).

These two mutant proteins also undergo electrophoretic retardation during SDS-PAGE when extracts are prepared from cells treated with LPS, even in the absence of ALLN (Fig. 5D). The extents of retardation for the mutant proteins were somewhat less than that of the WT protein, but this could be due to differences in conformation. These results suggest that the mutant proteins can be partially phosphorylated in response to stimulation but that phosphorylation at both serine 32 and serine 36 is required for the protein to be degraded (see Discussion). Finally, we constructed a mutant which encodes a protein in which both serines 32 and 36 are changed to alanines. This protein, when stably transfected into 70Z/3 cells, was also not degraded in response to LPS (Fig. 5E) or PMA (data not shown). In addition, this protein did not undergo any change in electrophoretic mobility when cells were treated with ALLN (Fig. 5E, last lane), indicating a complete absence of ligand-induced phosphorylation. The amount of transfected human IκBα was somehow variable depending on the mutant considered. We wondered whether this was an indication of a different stability of these mutants in unstimulated cells. Therefore, we measured the half-lives of these molecules by pulse-chase experiments in untreated cells and found that the half-lives of WT human and endogenous murine IκBα were about 45 min, while that of mutant S36A was more than 60 min (data not shown). Therefore, the amount of a given mutant protein in a given transfection is not a reflection of its half-life but is more likely to be random, as observed from the analysis of several transformation experiments (not shown).

In order to obtain more-direct evidence that phosphorylation takes place on these serines, we took advantage of our observation that thrombin cleaves human IκBα after amino acid 61 (at the sequence PRGS; see also reference 12), giving rise to an N-terminal fragment containing only two phosphorylatable residues, serines 32 and 36 (there is also a Y at position 42, but immunoblotting analysis with anti-phosphotyrosine antiserum indicated that IκBα is not phosphorylated on tyrosines following treatment with PMA or LPS). Cytoplasmic extracts of 70Z/3 clones, either untreated or stimulated with LPS in the presence of ALLN, were immunoprecipitated first with an antiserum raised against the C terminus of human IκBα. Digestion with thrombin was carried out on the protein A-Sepharose beads, therefore releasing the N-terminal 61 amino acids of this molecule into the supernatant. This supernatant was run on a Tricine gel (allowing resolution of small peptides), which was then transferred to Immobilon mem-

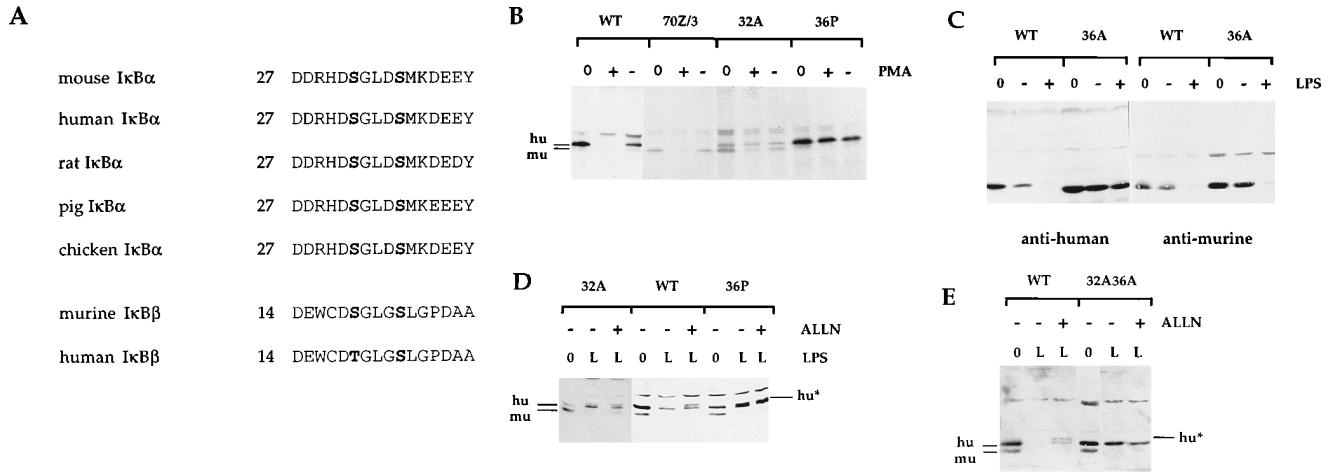

FIG. 5. (A) Amino acid sequences of IκBα proteins showing homology around serines 32 and 36 (shown in boldface). The corresponding sequences of murine (34) and human (16) IκBβ are also shown. (B) 70Z/3 cells (15×10^6) were metabolically labelled for 2 h with L-[^{35}S]cysteine–L-[^{35}S]methionine and then were diluted in prewarmed, complete medium containing 50 mM L-cysteine–L-methionine. Cells were then either lysed directly (0) or incubated for 20 min in the absence (−) or presence (+) of 50 ng of PMA per ml before lysis. Cells were lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.05% sodium deoxycholate), and after centrifugation for 2 min in a microcentrifuge, supernatants were transferred to new tubes. SDS was added to a final concentration of 2%, and samples were boiled before dilution in RIPA buffer. After preclearing overnight with a preimmune serum, IκBα proteins were precipitated with anti-IκBαN (1309), anti-IκBαC (296), and anti-rat IκBα (32) antisera and were analyzed by SDS-PAGE. Extracts were prepared from cells expressing the following: WT, human IκBα WT protein; 70Z/3, 70Z/3 cells transformed with the Rc-CMV vector alone; 32A, human IκBα protein containing the mutation serine 32 to alanine; 36P, human IκBα protein containing the mutation serine 36 to proline. (C) 70Z/3 cells expressing WT IκBα protein or the mutant protein carrying the mutation serine 36 to alanine (36A) were treated with 20 μg of cycloheximide per ml (−) or 20 μg of cycloheximide per ml plus 20 μg of LPS per ml (+) or were left untreated (0) for 1 h. The cells were pelleted, lysed in SDS-PAGE loading buffer, boiled, and loaded onto duplicate gels. After blotting, filters were probed with anti-human IκBα antiserum (1309; left panel), to detect only the transfected molecule, or anti-murine IκBα antiserum (751; right panel), to detect the endogenous murine molecule. (D) Cells expressing WT or mutant (32A or 36P) human IκBα proteins were grown to a density of 10^6 cells per ml and then were pelleted, resuspended at 5×10^6 cells per ml, and incubated for 45 min in the presence (+) or absence (−) of the proteasome inhibitor ALLN (100 μM). Cells were then treated with 10 μg of LPS (L) per ml for 45 min or left untreated (0), and cytoplasmic extracts were prepared and analyzed by Western blotting with the S7 anti-IκBα antiserum. The positions of the murine (mu), human (hu), and electrophoretically retarded human (hu*) proteins are indicated. (E) Cells expressing WT or mutant human IκBα protein containing the double mutation of serines 32 and 36 to alanines (32A36A) were grown to a density of 10^6 cells per ml and then were pelleted, resuspended at 5×10^6 cells per ml, and incubated for 45 min in the presence (+) or absence (−) of the proteasome inhibitor ALLN (100 μM). Cells were then treated with 10 μg of LPS (L) per ml for 45 min or left untreated (0), and cytoplasmic extracts were prepared and analyzed as described in the legend to Fig. 4D. The positions of the murine (mu), human (hu), and electrophoretically retarded human (hu*) proteins are indicated.

branes and blotted with antiserum 1309, raised against the N terminus of IκBα. The results shown in Fig. 6 demonstrate the presence of an additional band, with slower mobilities in the cases of the WT human molecule (lane 2) as well as the S36P mutant (lane 5) but not with slower mobility in the case of the double mutant S32A, S36A (lane 7). Treatment of the thrombin supernatant of the WT molecule with alkaline phosphatase

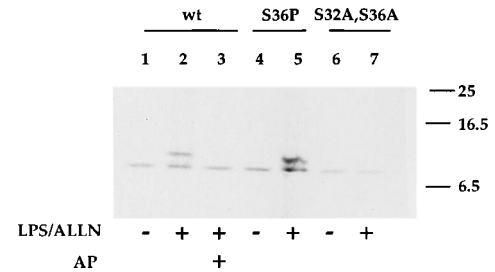

FIG. 6. Cytoplasmic extracts of 70Z/3 clones transfected with either WT human IκBα or with mutants S36P or S32A,S36A, either untreated (−) or stimulated with 10 μg of LPS for 45 min in the presence of 100 μM ALLN (+) as indicated, were boiled for 5 min in 1% SDS and then subjected to immunoprecipitation with antiserum 296, directed against the C terminus of IκBα, in the presence of phosphatase inhibitors. The immunoprecipitate was digested with thrombin as described in Materials and Methods, and the released N-terminal 61-amino-acid peptide was analyzed by electrophoresis on a 16.5% Tricine gel followed by immunoblotting with antiserum 1309, raised against the N terminus of human IκBα. In lane 3, the peptide was treated with alkaline phosphatase (AP) before analysis on the Tricine gel. Molecular mass markers (in kilodaltons) are on the right.

results in the disappearance of the upshifted band (lane 3), indicating that it is due to phosphorylation. These results demonstrate that induced phosphorylation takes place in the first 61 amino acids of the molecule, that is, on serine 32 or 36 or both (see Discussion). To verify that the stabilities of these mutants were not the result of a loss of ability to bind to Rel-family proteins, we tested their abilities to interact with either c-Rel (Fig. 7) or RelA (data not shown). Mutation of either serine 32 or 36 did not affect the affinity of the mutant proteins for c-Rel. The double mutant of serines 32 and 36 to alanines bound both c-Rel and RelA, with affinities comparable to that of the WT protein (data not shown).

DISCUSSION

Activation of NF-κB in vivo is dependent upon the proteolytic degradation of the inhibitor IκBα following stimulation, a process thought to take place in the context of the NF-κB/IκB complex following the phosphorylation of the IκBα molecule (1, 9, 18, 21, 23, 36). Here we demonstrate that the degradation of IκBα in response to extracellular stimulation is dependent upon the presence of an intact C terminus as well as the phosphorylation of serines 32 and 36. These results are in broad agreement with recently published data (6), with one important difference: in our cell line it is necessary to mutate both serines 32 and 36 to abolish ligand-inducible phosphorylation, whereas according to the published data, mutation of one serine or the other was sufficient. The reason for this discrepancy is not clear. More recently, two reports demon-

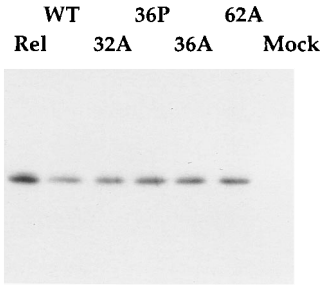

FIG. 7. Lysates from 293 cells transfected either with WT human IκBα or with human IκBα containing the mutation Ser-32 to Ala (Ser-32→Ala) (32A), Ser-36→Pro (36P), Ser-36→Ala (36A), or Ser-62→Ala (62A) or cells transfected with vector alone (Mock) were mixed with lysates from cells transfected with c-Rel and were metabolically labelled with L-[^{35}S]methionine–L-[^{35}S]cysteine and precipitated with anti-IκBαN antiserum (1309). As a positive control, a sample of the labelled lysate was precipitated with anti-c-Rel (Rel). Proteins were analyzed by SDS-PAGE; the presence of a band comigrating with that of the positive control indicates the ability of a given mutant of IκBα to interact with c-Rel.

strated the importance of serines 32 and 36 for the induced degradation of the IκBα molecule (5, 35). However, neither of these reports directly demonstrates that phosphorylation takes place on these two serines. One question that remains unanswered is whether in vivo phosphorylation takes place on both serines or on one of them only. The fact that the mutation of only one serine abolishes degradation but not phosphorylation on the other serine would tend to suggest that phosphorylation on both serines is required; however, direct sequencing or mass spectrometry analysis of the phosphorylated peptide isolated from stimulated cells will be necessary to answer this question.

The C terminus of IκBα is rich in the amino acids P, E, D, S, and T, and such PEST sequences have been correlated with high rates of protein turnover (25). Our studies have shown that the sequences between amino acids 279 and 290 are critical for degradation of IκBα in response to extracellular stimuli, results that are in accordance with a recently published paper (28). This region of the molecule contains 75% (9 of 12) PEST residues, indicating an important role for such sequences in the degradation of IκBα. 2D gel electrophoresis and ^{32}P labelling analyses comparing the WT molecule with a mutant lacking the PEST domain suggest that this C-terminal region is multiply and variably phosphorylated in unstimulated cells. One alternative interpretation of our results, although less likely, is that the deletion of the C-terminal region in the Δ268C mutant removes regulatory determinants that mediate distal phosphorylation events. Since the mutation of serines and threonines in this region has no effect upon protein stability following induction, the role of these phosphorylations remains unclear. It may be that the C terminus acts to correctly position the IκBα molecule upon the NF-κB complex so that it can be recognized by the degradation machinery following phosphorylation upon serines 32 and 36.

We propose that the IκBα molecule is phosphorylated by a kinase (or kinases) which phosphorylates residues 32 and 36. Upon treatment of cells with inducers of NF-κB, this activity is upregulated and the increased phosphorylation of IκBα targets the protein for rapid degradation. This activation could result from a direct increase in kinase activity, or alternatively, it could be that the kinase activity is constitutive and that in uninduced cells there exists a highly active phosphatase which removes the phosphate groups from serines 32 and 36. Upon induction, the activity of the phosphatase would be sharply downregulated, resulting in the appearance of a hyperphosphorylated form of IκBα which is then degraded. Such a hypothesis might explain how NF-κB is activated by treatment of cells with the phosphatase inhibitor okadaic acid (see reference 36 and references therein). Analysis of the N-terminal sequences for known kinase sites reveals that serines at positions 32 and 36 each lie within a consensus casein kinase II (CKII) site. It must also be noted that these residues do not form a site for the double-stranded RNA-induced kinase protein kinase R (PKR), an enzyme which has been suggested to be required for NF-κB activation by double-stranded RNA and viruses (14).

It is interesting to note that IκBβ, the second major isoform of IκB found in cells, also contains two serines (or one serine and one threonine for the human molecule) separated by 3 amino acids (Fig. 5A) whose sequence context resembles that of serines 32 and 36 of the α isoform (16, 34). However, while IκBβ is degraded in response to LPS, it does not respond to PMA. Whether these residues are required for the phosphorylation and degradation of IκBβ, and whether the same activity is responsible for the phosphorylation of both molecules, remains to be established.

The control of protein degradation by means of phosphorylation or dephosphorylation has been previously described, although in most cases the sequences responsible have not been elucidated. Phosphorylation of microtubule-associated protein 2 (MAP-2) by the cyclic AMP-dependent protein kinase inhibits calpain-induced hydrolysis of MAP-2 (13). The stabilities of the two yeast cyclin inhibitor proteins Far1 and Sic1 also appear to be regulated by phosphorylation (19). Sic1 is degraded in a ubiquitin-dependent manner, possibly as a result of phosphorylation (for a discussion, see reference 30).

The manner by which the phosphorylation of IκBα targets the molecule for degradation is unclear. It is intriguing that both N- and C-terminal domains play roles. In the case of the recombination activator protein RAG-2, the sequences responsible for phosphorylation and degradation are found in the C-terminal 90 amino acids, which encode a threonine (T-490) as well as a lysine-rich region (17). Instability is determined by phosphorylation of threonine 490. Degradation of the c-Mos proto-oncogene product in activated *Xenopus* eggs requires ubiquitination of lysine residue 34, which is dependent upon the dephosphorylation of a serine residue at position 3 (22). How phosphorylation and dephosphorylation act to induce ubiquitin-dependent protein degradation in these two cases is not known.

The recent findings that p105, the precursor protein for the 50-kDa subunit of NF-κB, requires the ubiquitin-26S proteasome pathway for its maturation and that inhibitors of p105 maturation also inhibit the degradation of IκBα suggest that IκBα is also degraded by this pathway (23). More recent data show that phosphorylated IκBα is a substrate for ubiquitin-dependent proteolytic degradation (1a). We note the presence of lysine residues at positions 21, 22, and 38, which could serve as sites for ubiquitin binding. We are currently testing the functions of these lysines.

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