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Signal-Induced Degradation of IκBα: Association with NF-κB and the PEST Sequence in IκBα Are Not Required

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Signal-induced degradation of IκBα via the ubiquitin-proteasome pathway requires phosphorylation on residues serine 32 and serine 36 followed by ubiquitination on lysines 21 and 22. We investigated the role of other regions of IκBα which may be involved in its degradation. Here we report that the carboxy-terminal PEST sequence is not required for IκBα signal-induced degradation. However, removal of the PEST sequence stabilizes free IκBα in unstimulated cells. We further report that a PEST deletion mutant does not associate well with NF-κB proteins but is degraded in response to signal. Therefore, we conclude that both association with NF-κB and a PEST sequence are not required for signal-induced IκBα degradation. Additionally, the PEST sequence may be required for constitutive turnover of free IκBα.

The NF-κB family of transcription factors have emerged as key regulators of genes important for immune and inflammatory responses (28, 66). NF-κB can consist of many different homo- and heterodimeric partners, including the proteins c-Rel, RelA, NF-κB1, NF-κB2, and RelB. NF-κB has been well conserved through evolution as a homologous protein called dorsal in *Drosophila melanogaster*, which is essential to dorsoventral patterning in the *Drosophila* embryo (61). All Rel/NF-κB proteins have an approximately 300-amino-acid (aa) stretch in the amino terminus known as the Rel homology domain, which is necessary for dimerization and DNA binding. The c-Rel, RelA, and RelB members contain potent transcription activating domains in their carboxy termini. NF-κB proteins can be oncogenic, as v-Rel, the resident oncogene of reticuloendotheliosis virus, causes fatal lymphomas in young birds (26). Additionally, suppression of RelA by antisense RNA regresses tumors in mice (31, 37). NF-κB also plays an important host role in the life cycles of many viruses, including human immunodeficiency virus type 1 (HIV-1) and human T-cell leukemia virus type 1.

NF-κB was first identified as a protein complex that bound to the enhancer region present in the intron of the immunoglobulin κ light-chain gene (60). Though constitutively nuclear in mature B cells, NF-κB was also found to exist in the cytoplasm of pre-B cells and virtually all other cell types. NF-κB cytoplasmic retention is through association with the inhibitor protein, IκBα (7). IκBα masks the nuclear localization sequence of NF-κB (12, 23). When cells are activated by various extracellular signals, including tumor necrosis factor alpha (TNF-α), interleukin-1α, lipopolysaccharide (LPS), and tetradecanoyl phorbol acetate, IκBα is phosphorylated and degraded rapidly. Subsequently, free NF-κB enters the nucleus and activates the transcription of many different target genes, such as the gamma interferon, IL-2, granulocyte-macrophage colony-stimulating factor, and major histocompatibility complex class I genes (for a review, see reference 28). IκBα degradation is essential for NF-κB activation, as several protease inhibitors that block IκBα degradation also inhibit nuclear transfer of NF-κB (20, 30). The biological role of IκBα is essential, since IκBα knockout mice die 6 to 8 days postnatally (13). The IκBα$^{-/-}$ mice have constitutive nuclear NF-κB proteins (p50 homodimer and p50/p65 heterodimer) in lymphoid cells, confirming the general notion that IκBα sequesters NF-κB proteins in the cytoplasm.

The IκBα protein contains ankyrin repeats which are also found in many other regulatory proteins, including NF-κB1 and NF-κB2 (11, 25, 41, 46, 66). Other IκB proteins (IκBβ, IκBγ, bcl3, and IκB-R [33, 49, 51, 64]) identified on the basis of their association with NF-κB also contain ankyrin repeats. IκBα and IκBβ are ubiquitously expressed, while other IκBs are more tissue restrictive. The products of the NF-κB1 and NF-κB2 genes, p105 and p100, have been shown to undergo proteolytic processing to yield DNA-binding subunits p50 and p52, respectively. The NF-κB1 protein is processed by the ubiquitin-proteasome system (50). Signal-induced degradation of IκBα has also been attributed to proteasomes (2, 19).

The molecular processes known to be involved in IκBα signal-induced degradation are as follows: (i) IκBα is phosphorylated on serine residues 32 and 36 (15, 16, 65); (ii) ubiquitination occurs on lysines 21 and 22 (8, 58); and (iii) the proteasome degrades IκBα, allowing NF-κB to be released and enter the nucleus (1, 21, 44). Other residues of IκBα may be involved in this processes. The IκBα carboxy terminus consists of a stretch of proline, serine, threonine, aspartate, and glutamate residues known as a PEST sequence. PEST sequences have been statistically identified as signals for protein instability (54), although the precise mechanism through which PEST sequences act is unknown. Two reports have suggested that the IκBα carboxy-terminal PEST sequence is required for signal-induced degradation of IκBα (16, 67). To address this question, we removed the IκBα PEST sequence. The lack of a PEST sequence generates a more stable IκBα protein. However, this stability is conferred only in the absence of signal. In contrast, we show that removal of the carboxy terminus is irrelevant to signal-induced degradation of IκBα. Lack of the PEST region also greatly reduces the affinity of NF-κB for IκBα, leading to a majority of free IκBα protein. Even so, this uncomplexed PEST-minus IκBα still undergoes phosphorylation and degradation in response to external signals. We therefore rule out two requirements for signal-induced IκBα degradation: (i) a PEST sequence and (ii) association with NF-κB.

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However, the PEST sequence plays an important role in the basal degradation of free IκBα.

MATERIALS AND METHODS

Cell culture and reagents. HeLa and 293 cells were grown in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and the antibiotics penicillin and streptomycin. 70/Z-CD14 cells stably expressing the LPS receptor have been described elsewhere (10). The cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS, 1 mg of G418 (Gibco-BRL) per ml, and 50 μM β-mercaptoethanol. Jurkat cells were grown in RPMI 1640 with 10% heat-inactivated FBS. TNF-α (Calbiochem) was used at 10 ng/ml, LPS (Sigma) was used at 1 μg/ml, and the phosphatase inhibitor calyculin A (Gibco-BRL) was used at 0.3 μM.

Plasmid construction. The IκBαΔPEST cDNA was constructed by PCR using a 5′ primer against the T3 promoter (Stratagene), a 3′ primer (5′-TATAAGC TTATTCCAAGGTCAGCTGGCT-3′), and the target plasmid pBluescript-murine IκBα. The product was digested with *Bam*HI and *Hin*dIII and cloned into the polylinker of pCMX-PI_2 (20). pRK5-murine IκBα and IκBαΔPEST were constructed by blunt insertion of the full-length or deleted IκBα cDNA into the polylinker of the parental expression vector, pRK5 (55). LXSN retroviral constructs (42) were also produced by blunt-end ligation into the polylinker of the retroviral vector. LHL-CA is a derivative of the retroviral construct LHL-SLX-CMV (56) in which the cytomegalovirus promoter has been replaced by the *Hpa*I-*Sma*I fragment of the cytomegalovirus–β-actin fusion promoter (47). IκBαΔPEST was blunt-end inserted into the polylinker to form LHL-CA-IκBαΔPEST. pCMX-IκBα and pCMX-RelA were previously described (20). pCMX-RelAΔC was generated by ligating the 1.2-kb *Sma*I fragment from pBluescript-RelA (48) into the Klenow enzyme-filled-in *Nhe*I polylinker site of the pCMX-PI_2. This manipulation deleted the last 137 aa and formed a termination codon. 3×κB-CAT contains the minimal thymidine kinase promoter from herpes simplex virus downstream of three different κB sites: murine immunoglobulin κ intronic enhancer site (GGGACTTTCC) (60), IL-2 receptor alpha-chain palindromic site (GGGAATTCCC) (9), and enhancer site from the murine IκBα promoter (GGGAATTTCC) (20). The κB enhancer region was constructed by producing the double-stranded oligonucleotide generated by Klenow enzyme fill-in of the synthetic oligonucleotide 5′-GGCGCATGCCAGGGGACTTTCC CAATGGGGAATTCCCCAATGGGGAAT T TCCC TC TCTAGAGCC-3′, with the primer 5′-GGCTCTAGAG-3′. The double-stranded oligonucleotide was digested with *Sph*I and *Xba*I and cloned into the *Sph*I-*Xba*I sites of pBLCAT2 (40). 3×MκB-CAT contains the following mutations (in boldface) to the foregoing sequences: immunoglobulin κ, **ATC**ACTTTCA; IL-2 receptor alpha-chain palindrome, **ATC**AATTCCA; and IκBα promoter enhancer, **ATC**AATTTCA. Construction of the mutant reporter plasmid was the same as for the wild-type reporter.

Transfections and production of stable pools of cells. 293 cell transfections were performed by the calcium phosphate method (68). Briefly, DNA was mixed in 0.625 M $CaCl_2$ and then added to an equal volume of 2× *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered saline solution (560 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4 [pH 7.12]). The mixture was added to 10^6 cells in 10 ml of medium or 3×10^5 cells in 3 ml of medium and incubated for 5 h at 5% CO_2. The medium was then changed, and the cells were allowed to recover overnight. Production of virus for the infection of Jurkat and HeLa cells was performed by cotransfection of 293 cells with the SIN-amphotropic helper plasmid (45) and the LXSN retroviral vector carrying the cDNA for either IκBα or IκBαΔPEST. After 48 h, the virus was collected, filtered, and added to 10^6 cells with Polybrene (4 μg/ml) for 6 h. The cells were then incubated in complete medium and allowed to recover for 48 h. G418 (Gibco-BRL) was then added at 500 μg/ml for HeLa cells and 1 mg/ml for Jurkat cells. Stable pools were collected after selection for about 1 week and maintained in the same doses of G418. Infection of 70Z/CD14 cells was performed by cocultivation with virus-producing 293 cells. 293 cells were transfected with the LHL-CA-IκBαΔPEST retroviral vector and the SIN-ecotropic helper plasmid (45) and cultivated alone for 24 h. 293 medium was removed, and 10^4 70Z/CD14 cells were added to the 293 cells in 5 ml of RPMI 1640 plus 10% FBS and Polybrene (4 μg/ml) without β-mercaptoethanol or G418 for 48 h. The 70Z/CD14 cells were removed and allowed to recover for 48 h. Hygromycin (1 mg/ml; Boehringer Mannheim), G418, and β-mercaptoethanol were then added, and after 1 week of selection, a stable pool was collected and maintained at 1 mg of hygromycin per ml.

Metabolic labeling and immunoprecipitation. Transfected 293 cells were washed twice in phosphate-buffered saline solution (PBS) and incubated for 1 h in 4 ml of cysteine- and methionine-free Dulbecco's modified Eagle medium supplemented with 10% dialyzed FBS (Gibco-BRL). Labeling was performed for 2 h with 0.5 mCi of [^{35}S]methionine (Du Pont-NEN) per ml, after which the cells were washed three times in complete medium and then chased for defined times with complete medium. Cell pellets were frozen on dry ice for later manipulation. After thawing, whole cell lysis was performed by adding radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris base [pH 8.8], 100 mM NaCl, 0.2% sodium deoxycholate, 0.2% Nonidet P-40, 0.2% Triton X-100) plus 0.5% sodium dodecyl sulfate (SDS) and the protease inhibitors aprotinin (2.3 μg/ml; Sigma) and phenylmethylsulfonyl fluoride (1 mM; Sigma). DNA was sheared by 10 passes through a 20-gauge needle. After boiling for 10 min, the lysates were measured for trichloroacetic acid-precipitable counts. The SDS was diluted to 0.1% with RIPA buffer, and the lysates were precleared for 1 h at 4°C with protein A-Sepharose (Pharmacia). The cleared lysates were normalized for labeling efficiency and incubated for 12 h at 4°C with protein A-Sepharose and IκBα antiserum (43). The pellets were washed in RIPA buffer, SDS sample buffer was added, and the pellets were boiled for 10 min prior to SDS-polyacrylamide gel electrophoresis (PAGE).

HeLa cell labeling and immunoprecipitation were done as for 293 cells except for the following steps. The labeling period for HeLa cells was 4 h. Whole cell lysis was performed under nondenaturing conditions, which consisted of lysis in RIPA buffer without SDS. RelA-containing complexes were then coimmunoprecipitated overnight with RelA antibody (43). To reduce background, coimmunoprecipitation was always followed by a second round of immunoprecipitation. The RelA antibody–protein A-Sepharose pellets were boiled for 10 min in RIPA buffer containing 100 μg of bovine serum albumin (BSA) per ml and 0.5% SDS. The SDS was diluted to 0.1% with RIPA buffer containing 100 μg of BSA per ml. IκBα antibody and protein A-Sepharose were added back to isolate RelA-associated IκBα. To isolate the free IκBα, the supernatants from the original RelA coimmunoprecipitates were incubated with IκBα antibody and protein A-Sepharose. These secondary coimmunoprecipitations were treated identically to the RelA-associated pellet. After the immunoprecipitation, pellets were washed in RIPA buffer, SDS sample buffer was added, and samples were boiled and run simultaneously on an SDS–12.5% polyacrylamide gel.

In vitro association analysis. In vitro transcription-translation was performed by using the T7 promoter contained within the pCMX vectors of RelA, IκBα, and IκBαΔPEST with rabbit reticulocyte lysate and [^{35}S]methionine, using a TNT kit as instructed by the manufacturer (Promega). Each protein was translated individually and mixed together in 150 μl of RIPA buffer containing BSA and the protease inhibitors aprotinin and phenylmethylsulfonyl fluoride. Association was carried out on ice for 1 h, and then 800 μl of RIPA buffer containing BSA and protease inhibitors was added along with RelA antiserum and protein A-Sepharose. The samples were tumbled for 4 h at 4°C and then washed once with 1 ml of RIPA buffer. Loading buffer was added, and the samples were run on SDS–12% polyacrylamide gels.

CAT assay. 293 cells (10^5) were transfected on six-well plates with the indicated expression and reporter constructs and pRSV-βgal in a total of 5 μg. After transfection, the cells were grown for 48 h, at which time they were washed once in PBS, scraped, and frozen on dry ice. Cell lysates were normalized for transfection efficiency by β-galactosidase activity. Chloramphenicol acetyltransferase (CAT) activity was then measured by using thin-layer chromatography followed by exposure of the plates to a PhosphorImager (Molecular Dynamics) and quantitation with Imagequant software.

Electrophoretic mobility shift assays. 293 cells were transfected on 60-mm-diameter plates with the indicated amounts of expression plasmid in a total of 10 μg of DNA and grown for 24 h. Cell fractionation was then performed by the micropreparation technique (3), and gel shift analysis was performed as described previously (34). Briefly, 5 μg of nuclear protein extract was incubated with 0.5 μg of poly(dI-dC) on ice for 20 min. A ^{32}P-labeled oligonucleotide containing the κB site from the HIV-1 long terminal repeat (18) was then added, and the mixture was incubated at room temperature for 30 min. The resulting complexes were resolved on a 4% nondenaturing polyacrylamide gel.

Cell stimulation and Western blot (immunoblot) analysis. In all cases, 10^7 cells were treated with the appropriate stimulus for the indicated times. The cells were washed once in PBS and frozen on dry ice. Cytoplasmic extracts were made as previously described (44). Fifty micrograms of protein was separated on SDS–15% polyacrylamide gels and transferred to nitrocellulose membranes (Micron Separations, Inc.). IκBα antibody was diluted 1:1,000 in 0.2% Tween–PBS with 5% nonfat milk and incubated with the membrane overnight. Horseradish peroxidase-conjugated donkey anti-rabbit antibody (Amersham) was diluted 1:3,000 and incubated with the membrane for 2 h. The IκBα-specific bands were then resolved by using a Renaissance detection kit (Dupont) as instructed by the manufacturer.

RESULTS

Removal of the PEST sequence stabilizes free IκBα. To test the function of the IκBα PEST sequence, an IκBα deletion mutant was generated. Figure 1A shows a diagram of mouse and human IκBα proteins and the region containing the PEST sequence. The position of the C-terminus deletion which generated mouse IκBαΔPEST (Δ39) is shown. The positions of equivalent mutants generated by others (16, 67) in human IκBα are also indicated. We first performed pulse-chase immunoprecipitation analysis on 293 cells transfected with expression constructs for IκBα and the deletion mutant IκBαΔPEST. Figure 1B shows the typical degradation profile which has been reproduced in many experiments. The half-life of IκBα analyzed in this manner is very short and has previ-

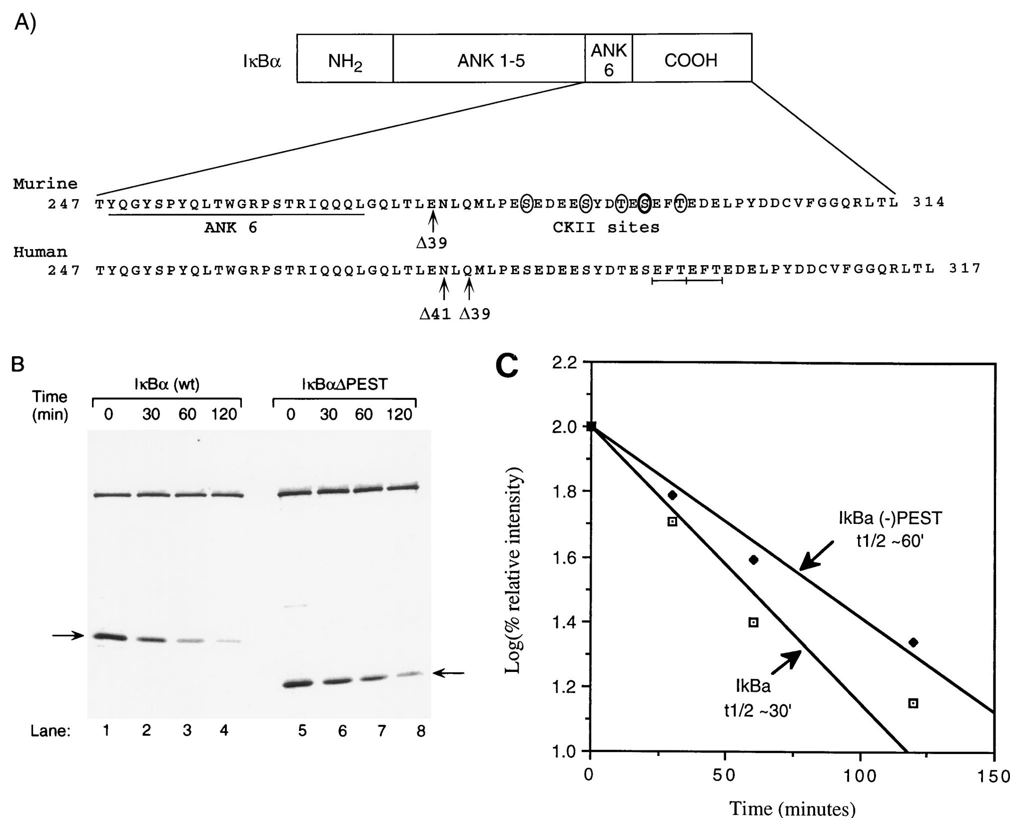

FIG. 1. Role of carboxy-terminal PEST sequence. (A) PEST sequences of murine and human IκBα proteins and the sequence of the ankyrin (ANK) repeat 6 domain. CKII sites are circled, with serine 293 in a boldface circle, and the EFT repeat found in human but not murine IκBα is underlined. Aside from the EFT repeat, the human and murine sequences within this region are identical. The PEST score given to this domain by the statistical computer program PESTfind (developed by S. Rogers and M. Rechsteiner) is a +7.79, which is considered high. The location of the last residue remaining in the murine IκBαΔPEST truncation as well as truncations of human IκBα constructed by other groups (16, 67) are indicated by arrows. (B) Time course analysis of free IκBα and IκBαΔPEST degradation without stimulus. 293 cells were transfected with 0.5 μg of pRK5-IκBα and 0.1 μg of pRK5-IκBαΔPEST. Twenty-four hours later, the cells were pulse-labeled with [^{35}S]methionine and chased for the indicated times. Lysates were normalized by trichloroacetic acid-precipitable counts. IκBα (lanes 1 to 4) and IκBαΔPEST (lanes 5 to 8) were immunoprecipitated with IκBα-specific antiserum and separated by SDS-PAGE (12.5% gel). Shown is the readout from a PhosphorImager. The arrows indicate the migration of IκBα (wild type [wt]) and IκBαΔPEST. (C) Quantitation of IκBα and IκBαΔPEST half-lives. The intensities of the bands corresponding to IκBα and IκBαΔPEST in panel B were measured from the PhosphorImager, using Imagequant software. The data were converted into percent relative intensity by assigning the time zero band of each time course as 100% and assigning the remaining time points a percentage that correlated to the fraction of its band and the time zero band. The log of each percentage was then calculated and plotted against time (in minutes) to generate the graph. ′, minutes.

ously been reported to be about 30 min (52, 63). The intensities of the bands corresponding to IκBα and IκBαΔPEST from Fig. 1B were used to quantitate the half-life of each protein (Fig. 1C). In our assay, the half-life of full-length IκBα is also 30 min (Fig. 1B, lanes 1 to 4, and C). When the PEST sequence is removed, the half-life increases to about 60 min (Fig. 1B, lanes 5 to 8, and C). In fact, because of the increased stability of IκBαΔPEST, five times more IκBα expression plasmid than IκBαΔPEST had to be transfected in order to obtain equal protein levels at time zero. When IκBα is cotransfected with NF-κB, the complexed IκBα half-life increases to over 4 h (52, 63). Because our full-length IκBα has a half-life similar to that found for IκBα transfected alone, we believe that the majority of the labeled pool of IκBα is not complexed with NF-κB. Thus, free IκBα is very unstable but can be made two times more stable by removal of its PEST sequence.

Removal of the PEST sequence destabilizes the NF-κB/IκBα complex. The IκBα PEST sequence plays a role in the degradation of free IκBα, but is the PEST required for signal-induced degradation? To assess the ability of a PEST deletion mutant to undergo signal-induced degradation, we chose an assay system involving transient transfection of three κB sites linked to a minimal promoter driving CAT. While optimizing the system, we were surprised to find that IκBαΔPEST could not inhibit exogenous RelA activity. Figure 2A, lane 9, shows the amount of activation seen when RelA was transfected alone. Titration of the IκBα expression constructs used for Fig. 1B in combination with a constant amount of RelA shows that at levels of plasmid in which the full-length IκBα almost completely inhibits CAT production (lanes 3 to 5), IκBαΔPEST has little or no effect (lanes 6 to 8). Previous reports have suggested that the ankyrin repeats, but not the PEST sequence, of IκBα are required for association and cytoplasmic retention of NF-κB (16, 22, 38). However, our data suggest that removal of the PEST sequence weakens the affinity of IκBα for NF-κB.

To investigate the idea that IκBαΔPEST does not associate well with NF-κB, we performed gel shift analysis. 293 cells were cotransfected with a constant amount of RelAΔC, which is missing the RelA transactivation domain, and various amounts of either IκBα full-length or IκBαΔPEST. We used RelAΔC because although it can dimerize, associate with IκBα, and bind to DNA (12, 23), it will not overproduce endogenous IκBα by virtue of the NF-κB/IκBα autoregulatory loop (17, 20, 63). Thus, we eliminated the effects of endogenous IκBα. Figure 2B shows that nuclear extracts from cells transfected with RelAΔC exhibit binding to κB sites (lane 1).

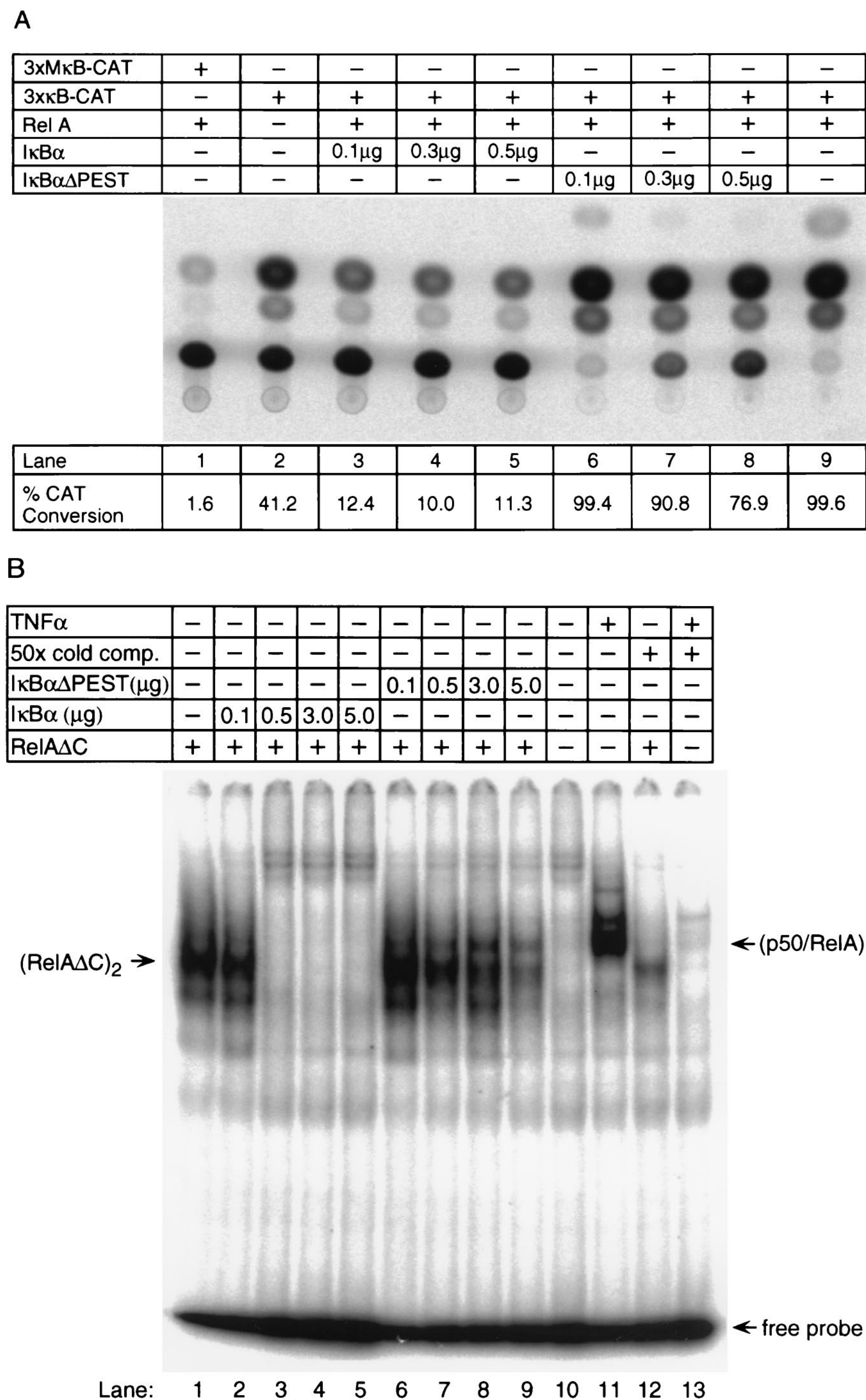

FIG. 2. IκBαΔPEST cannot inhibit activity of RelA. (A) IκBα, but not IκBαΔPEST, can inhibit RelA transactivation. CAT assays were performed on 293 cells transfected with the reporter and expression constructs indicated at the top. Aliquots of 0.5 μg of pCMX-RelA and the CAT reporter constructs were transfected where indicated. Cells were collected 48 h after transfection, and CAT assays were performed on normalized lysates. Thin-layer chromatography plates were exposed to a PhosphorImager, and the percent conversion shown at the bottom was quantitated with Imagequant software. The data are representative of three experiments performed with similar results. Lanes 1, cotransfection of pCMX-RelA and mutant 3×κB-CAT reporter; 2, transfection of wild-type 3×κB-CAT reporter alone; 3 to 5, wild-type reporter, constant amount of pCMX-RelA, and increasing amounts of pRK5-IκBα; 6 to 8, wild-type reporter, constant amount of pCMX-RelA, and increasing amounts of pRK5-IκBαΔPEST; 9, cotransfection of wild-type reporter and pCMX-RelA. Shown is the readout from the PhosphorImager. (B) IκBαΔPEST cannot retain RelAΔC in the cytoplasm. Electrophoretic mobility shift assay was performed on 293 cells transfected with the indicated plasmid amounts or stimulated with 10 ng of TNF-α per ml for 15 min. Three micrograms of pCMX-RelAΔC was transfected where indicated. Nuclear extracts were prepared, and gel shift was performed with the κB sequence from the HIV-1 long terminal repeat as a probe. The shift corresponding to RelAΔC homodimer is indicated (lane 1), as well as the endogenous NF-κB shift present upon TNF-α induction (lane 11). Lanes 2 to 6, constant amount of pCMX-RelAΔC and increasing amounts of pRK5-IκBα; lanes 6 to 9, constant amount of pCMX-RelAΔC and increasing amounts of pRK5-IκBαΔPEST; lane 10, 293 cells without transfection or stimulation; lanes 12 and 13, samples from lanes 1 and 11, respectively, in the presence of a 50-fold excess of unlabeled competitor (cold comp.).

This κB binding is abolished with cotransfection of increasing amounts of IκBα (lanes 2 to 5). In contrast, transfection with IκBαΔPEST has a greatly reduced effect on κB binding (lanes 6 to 9). Although equal levels of plasmid were transfected, the amount of IκBαΔPEST protein is much higher than the amount of IκBα because of its increased stability (Fig. 1B). Figure 2B also includes several controls with excess unlabeled oligonucleotide (lanes 12 and 13) and induction of endogenous NF-κB (p50/RelA) with TNF-α (lane 11). To be certain that cytoplasmic contamination of overexpressed IκBα was not responsible for the inhibition of NF-κB binding seen in lanes 2 to 5, we performed deoxycholate–Nonidet P-40 treatment of the nuclear extracts followed by gel shift and found no difference in the binding profile seen in Fig. 2B (data not shown). Western blot analysis was used to confirm that the various plasmids used for Fig. 2A and B were coexpressing at expected levels (data not shown). Thus, these data combined with the transactivation data (Fig. 2A) suggest that IκBαΔPEST is unable to associate with NF-κB proteins and thus has no effect on their role in transcriptional activation.

IκBαΔPEST does not associate efficiently with NF-κB in a stable cell line or in vitro. The levels of transiently transfected IκBα are much greater than endogenous levels. Therefore, to study IκBαΔPEST in a more native system, we generated HeLa cells stably expressing the murine wild-type IκBα and IκBαΔPEST. We investigated the amount of IκBα and IκBαΔPEST associated with RelA by metabolically labeling the cells with [^{35}S]methionine followed by immunoprecipitation with RelA antibodies. The RelA immunoprecipitation complexes were boiled, and IκBα proteins present were immunoprecipitated with IκBα antibodies. Figure 3A shows that in HeLa cells, the IκBα protein can be identified (lane 1, hIκBα). Since murine and human IκBα protein have different mobilities in SDS-PAGE, in an IκBα-infected cell line (lane 2), both human and mouse IκBα proteins can be identified. However, in lane 3, which contains HeLa cells stably infected with IκBαΔPEST mutant, the human IκBα protein is readily detected but IκBαΔPEST protein is hardly detectable.

To determine the levels of unassociated IκB proteins, supernatants from the RelA immunoprecipitations were further immunoprecipitated with IκBα antibodies (lanes 4 to 6). Only the RelA supernatants from the IκBαΔPEST stable cell line contained substantial amounts of unassociated protein (lane 6). Control immunoprecipitations of the associated and free samples with RelA antiserum showed that RelA was completely cleared after the first round of immunoprecipitation (data not shown). Because in HeLa cells IκBα appears to coimmunoprecipitate almost entirely with RelA antiserum (Fig. 3A; compare lanes 1 and 4), we conclude that the majority of IκBαΔPEST protein is in free form rather than in association with other NF-κB proteins. Free IκBαΔPEST does not appear to be caused by a saturation of RelA, as the amount of exogenous murine IκBα associated with RelA is much higher than the amount of IκBαΔPEST (lanes 2 and 3). Instead, it seems to be due to a lower affinity to RelA. To test this directly, we performed an in vitro association assay in which in vitro-translated RelA was mixed with increasing amounts of IκBα and IκBαΔPEST and the resulting complexes were immunoprecipitated with RelA antiserum (Fig. 3B). Comparison of lanes 5 to 7 and lanes 8 to 10 shows that IκBα, but not IκBαΔPEST, can readily form complexes with RelA. In fact, only at the highest amount of IκBαΔPEST used can any of this protein be seen associated with RelA (lane 10). Lane 11 shows the specificity of the antibody by competition with excess peptide from which the antibody was raised. Lanes 1 to 3 show the similarity in amounts of protein present in 1 μl of the in

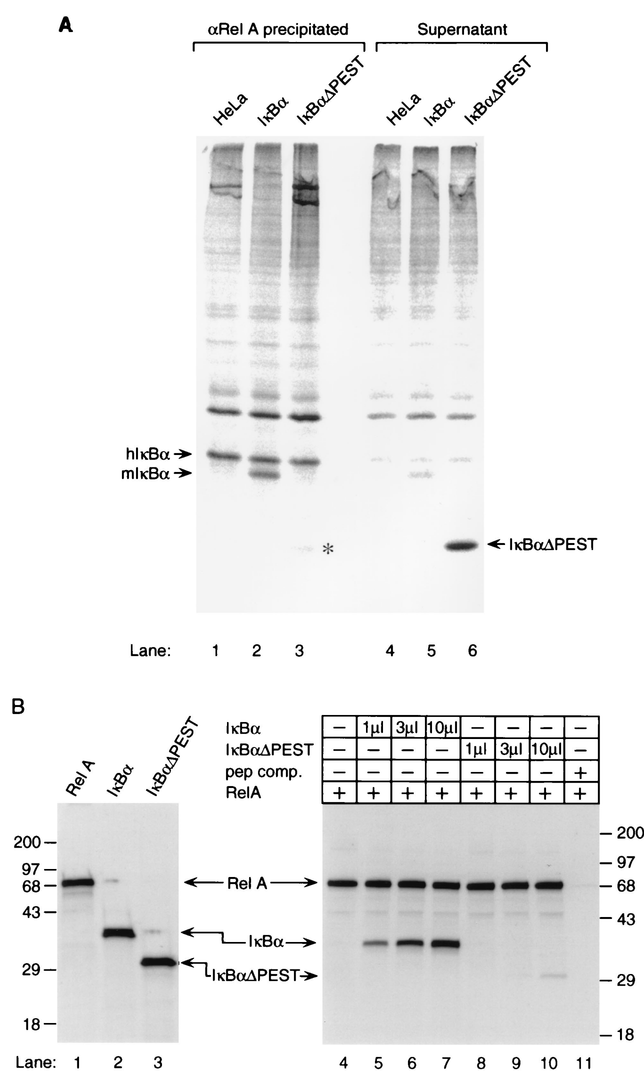

FIG. 3. IκBαΔPEST does not associate well with RelA complexes in stable HeLa cell lines or in vitro. (A) HeLa cells alone or stably expressing IκBα or IκBαΔPEST were analyzed for the amounts of IκBα protein in complex with RelA. Cells were labeled with [^{35}S]methionine, lysates were normalized by trichloroacetic acid-precipitable counts, and RelA-associated proteins were coimmunoprecipitated. The RelA complexes were then boiled, and IκBα proteins were immunoprecipitated (lanes 1 to 3). To analyze the uncomplexed IκBα pools, IκBα antiserum was added back to the supernatant from the primary coimmunoprecipitation. These complexes were also boiled and immunoprecipitated again with IκBα antiserum (lanes 4 to 6). The immunoprecipitates were separated by SDS-PAGE (12.5% gel) and visualized with a PhosphorImager. Locations of human and murine IκBα (hIκBα and mIκBα) are indicated by arrows on the left, and the position of IκBαΔPEST is indicated by the arrow on the right. The small amount of IκBαΔPEST associated with RelA is indicated by the asterisk (lane 3). (B) In vitro association of RelA with IκBα and IκBαΔPEST. Individually in vitro-translated RelA, IκBα, and IκBαΔPEST were mixed together in various combinations and coimmunoprecipitated with RelA antiserum. Lanes 1 to 3 show the protein output from 1 μl of each transcription-translation reaction mixed with load buffer and directly loaded for SDS-PAGE. Lane 4 contains only RelA (1 μl) immunoprecipitate, and lane 11 is the same except that the antibody was preincubated with excess peptide against which the antiserum was raised. Lanes 5 to 7 contain 1 μl of RelA and the indicated increases in IκBα protein. Lanes 8 to 10 contain 1 μl of RelA and the indicated amounts of IκBαΔPEST protein. Bands corresponding to RelA, IκBα, and IκBαΔPEST are labeled with arrows indicating their positions. Molecular size markers (in kilodaltons) are shown to the side of each gel. All samples were subjected to SDS-PAGE (12% gel), dried, and visualized by autoradiography. pep comp., peptide competitor.

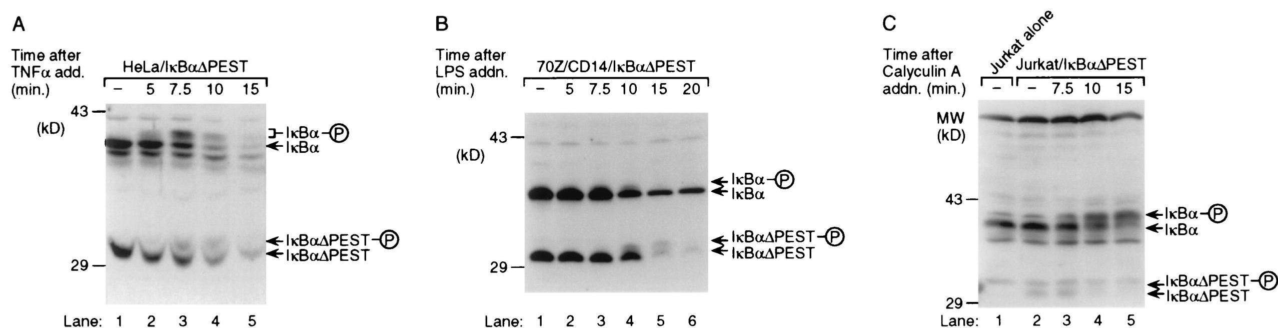

FIG. 4. Degradation of IκBα and IκBαΔPEST in HeLa, 70Z/CD14, and Jurkat stable cell lines upon stimulation. (A) Time course analysis of the modification and degradation of IκBα and IκBαΔPEST in HeLa cells stimulated with TNF-α. HeLa cells stably expressing the IκBαΔPEST protein were stimulated with 10 ng of TNF-α per ml for the indicated times. Cytoplasmic extracts were prepared, and 50 μg of protein was separated by SDS-PAGE (15% gel). Proteins were immobilized onto a nitrocellulose membrane, and IκBα proteins were visualized by using IκBα antiserum. The locations of endogenous human IκBα and IκBαΔPEST as well as the phosphorylated forms of each are indicated on the right. (B) Analysis of LPS stimulation in 70Z/CD14 murine pre-B cells stably expressing IκBαΔPEST. Cells were stimulated with 1 μg of LPS per ml for the indicated times and treated as for panel A. The locations of endogenous IκBα and IκBαΔPEST and their phosphorylated forms are indicated on the right. (C) Analysis of calyculin A-stimulated Jurkat T cells stably expressing IκBαΔPEST. Cells were stimulated with 0.3 μM calyculin A for the indicated times and treated as for panel A except that 75 μg of cytoplasmic extract was loaded for SDS-PAGE. Lane 1, extract from Jurkat cells without IκBαΔPEST; lanes 2 to 6, extracts from Jurkat/IκBαΔPEST cells. The locations of endogenous IκBα and IκBαΔPEST and their phosphorylated forms are shown on the right. The locations of molecular mass markers are shown on the left.

vitro-translated lysate. We cannot rule out the possibility that the IκBαΔPEST/NF-κB complex may have been disrupted during the coimmunoprecipitation procedure. But the combined data in Fig. 2 and 3 lead us to conclude that removal of the PEST sequence destabilizes the IκBα/NF-κB complex. This reduced affinity for NF-κB is in accordance with in vitro work which showed that deletion of the IκBα C terminus leads to a loss of gel shift inhibition (22, 29, 35, 38). Therefore, the additional affinity required to strip NF-κB from DNA may come from contact through the PEST sequence.

Signal-dependent degradation of IκBαΔPEST. Since IκBαΔPEST does not efficiently associate with RelA, we wanted to study how this would effect its degradation in response to external signals. Pools of stable cells expressing the IκBαΔPEST protein were generated by retroviral infection of HeLa, 70Z/CD14, and Jurkat cells. Each cell line was subjected to a specific stimulus followed by Western blot analysis of cytoplasmic extracts with IκBα-specific antibodies (Fig. 4). In HeLa cells treated with TNF-α (Fig. 4A), the endogenous human IκBα is rapidly modified (lanes 2 to 4) and degraded by 15 min postinduction. The modification and degradation kinetics of IκBαΔPEST are very similar to those of the endogenous IκBα, supporting the claim that removal of PEST sequences from IκBα does not compromise its ability to be (i) modified and (ii) degraded in response to external signal.

To ensure that the modification and degradation pattern of IκBαΔPEST is not cell type specific, we carried out similar experiments with a murine pre-B-cell line (70Z/CD14) and Jurkat T cells. Data in Fig. 4B and C shows that the pattern of degradation of IκBαΔPEST, in either stably infected 70Z/CD14 or Jurkat cells, was essentially the same as that observed for the endogenous IκBα protein. In fact, IκBαΔPEST protein appears to degrade more completely than endogenous IκBα in LPS-treated 70Z/CD14 cells (Fig. 4B; compare lanes 4 to 6). Thus, from data obtained for three different cell lines stably infected with IκBαΔPEST, we conclude that signal induced degradation of IκBα does not require the C-terminal PEST sequences.

DISCUSSION

Following stimulation, one of the first genes to be turned on by NF-κB protein is the IκBα gene (66). The newly synthesized IκBα is phosphorylated by casein kinase II (CKII) and binds to NF-κB freed by degradation of IκBα or proteolytic cleavage of NF-κB1 and NF-κB2 complexes. In this manner, new NF-κB/IκB complexes are formed until the next signal is transduced to the cell. Thus, there is a very tight regulation of NF-κB/Rel proteins largely controlled by IκBα. For example, if IκBα is overproduced, such as upon the addition of glucocorticoid, the NF-κB activation is severely hindered or blocked, leading to suppression of genes involved in inflammatory response (6, 57). Additionally, newly synthesized IκBα protein traverses to the nucleus, where it either removes NF-κB proteins bound to the DNA or creates a nuclear pool to prevent future rounds of stimulation (5). Thus, excess IκBα in the cell poses a substantial impediment to its normal function.

How does the cell maintain this exquisite regulation of IκBα? How do the different regions of the IκBα contribute to the regulation? Topologically, the IκBα protein can be divided into three domains, the N terminus (72 aa), the ankyrin repeats (aa 73 to 242 for five ankyrin repeats and aa 247 to 269 for the incomplete sixth ankyrin repeat), and the carboxy terminus (aa 270 to the end). Each domain has an important role in NF-κB signaling pathway. However, the function of C-terminal domain is more subtle.

The NH_2 terminus. Several reports have shown that upon induction, serine residues 32 and/or 36 in the N-terminal domain are phosphorylated, followed by ubiquitination on lysine residues 21 and 22. The multiubiquitinated IκBα complexed with NF-κB/Rel proteins is then degraded by the proteasome (2, 19). If serine 32 or 36 is mutated to alanine, the IκBα protein is neither phosphorylated nor degraded in response to signal (15, 16, 65). Similarly mutation of lysines 21 and 22 to arginine, though allowing phosphorylation of IκBα, severely reduced ubiquitination and subsequent degradation (8, 58). Thus, it is clear that the N terminus of IκBα is essential for signal-induced modification, a prerequisite for its degradation. Although the structure of IκBα alone or in association with κB protein is not yet known, biochemical and genetic data suggest that the N-terminal domain is unassociated and accessible for phosphorylation by kinase(s). Our results show that IκBαΔPEST, which poorly associates with RelA (Fig. 3), is still capable of being phosphorylated upon signal induction (Fig. 4). Furthermore, Chen et al. (19) have shown that IκBαΔC mutant (missing 75 aa at the C terminus) can be not only

phosphorylated but also ubiquitinated in response to a signal. It thus appears that N-terminal domain may be sufficient for both signal-induced hyperphosphorylation and subsequent ubiquitination.

The ankyrin repeats. It has been well established that the ankyrin repeats in IκBα are required for association with NF-κB/Rel protein. Extensive mutational analyses have shown that nearly all five, and possibly the sixth incomplete, ankyrin repeats participate in protein-protein interaction (35, 65a). The ankyrin repeats also define the specificity that IκB protein has for different members of the NF-κB/Rel family (38). Several investigators (4, 19, 53, 62) have reported that carboxy-terminal deletions beyond the PEST sequence, including ankyrin repeat 6, can prevent signal-induced IκBα degradation. It will be interesting to determine whether residues within this domain also contribute to IκBα signaling.

The C terminus. There are two immediately recognizable landmarks in the C-terminal domain of IκBα: the PEST sequence and the CKII phosphorylation sites. PEST sequences have been shown to be involved in protein stability (54), and therefore it is logical to study the mechanism of degradation of IκBα. In fact, several investigators have recently shown that signal-induced degradation of IκBα requires not only the N-terminal domain but also a PEST-containing C-terminal domain. Brown et al. (16) have shown that in stable EL-4 murine T cells, a 41-aa carboxy-terminal deletion of human IκBα (Fig. 1) undergoes phosphorylation but not significant degradation. Similarly, Whiteside et al. (67) have reported that a C-terminal 39-aa deletion mutant of IκBα (Fig. 1) in stable 70Z murine pre-B cells does not undergo signal-induced degradation. These data are in direct disagreement with the results presented here (Fig. 4). We show that IκBαΔPEST (39 aa deleted from the C terminus of IκBα [Fig. 1]) stably expressed in three different cell lines undergoes signal-induced phosphorylation and degradation. Our results are, however, in agreement with those of Aoki et al. (4) and Sun et al. (62), who have shown that mutants in which the IκBα PEST sequence is deleted (similar to IκBαΔPEST used here) undergo TNF-α-induced degradation similarly to endogenous IκBα. At present we are unable to resolve the discrepancy between the results of Aoki et al. (4), Sun et al. (62), and ourselves and those of Brown et al. (16) and Whiteside et al. (67).

Additionally, using in vitro analysis or transient overexpression, Brown et al. (16), Ernst et al. (22), and Sun et al. (62) have reported that deletion of the IκBα PEST sequence does not disrupt its physical association with NF-κB. However, using stable cell lines and in vitro association, we have shown that IκBαΔPEST shows rather weak binding to NF-κB/Rel proteins (Fig. 3A and B). Furthermore, this C-terminally truncated protein is unable to inhibit either the transactivation by RelA (Fig. 2A) or transfer of RelA to the nucleus, as judged by gel shift analysis (Fig. 2B). It has previously been shown by us and others (29, 36, 38) that the C-terminal region of IκBα may directly associate with NF-κB/Rel proteins in a manner such that the N terminus is free but the remainder of the IκBα protein is in direct contact. The C terminus is therefore required for strong binding affinity and possibly for interaction with the region of the κB proteins directly contacting DNA.

PEST is needed for basal degradation of free IκBα. Most of the studies on NF-κB/Rel regulation have focused on the degradation of IκBα. Very little free IκBα is found in cells (Fig. 3), and it is well established that free IκBα is more unstable than bound IκBα (52, 63). Thus, it appears that cells have evolved an efficient way to clear free IκBα. Figure 1B shows that the half-life of free IκBα is around 30 min. However, the half-life of IκBαΔPEST is increased to 60 min, suggesting that PEST sequences may play a direct role in the degradation of free IκBα. Interestingly, in Fig. 3 (lane 6), there is a considerable amount of free IκBαΔPEST, which further suggests a role of PEST sequences in degradation of free IκBα. We have previously shown that serine 293, part of the PEST sequence, is preferentially phosphorylated by CKII, but if this serine is mutated to alanine, neighboring serine and threonine residues undergo compensatory phosphorylation (10, 59). A mutant in which all five possible CKII phosphorylation sites (Ser-283, Ser-288, Thr-291, Ser-293, and Thr-296) within the PEST sequence are mutated to alanine (Mut F) has a half-life twice that of wild-type IκBα (59). Thus, a plausible mechanism for degradation via the PEST sequence would evoke recognition of PEST phosphorylation by a protease system. This recognition would be restricted to free IκBα. We therefore conclude that the PEST sequence and its phosphorylation play an important role in signal-independent degradation of free IκBα.

Does the free IκBα undergo basal degradation via proteasomes like complexed IκBα? Data presented here (Fig. 4) and by others (4, 19) have shown that IκBαΔPEST can undergo phosphorylation and ubiquitination in a signal-dependent manner and likely degrades via proteasomes. However, addition of several proteasome inhibitors (e.g., ZLLF or Calpain I inhibitor) have little or no effect on the stability of free IκBα, whereas chloroquine, a lysomal inhibitor, stabilized IκBα for over 3 h (65a). Chloroquine, however had no effect on signal-induced IκBα phosphorylation or degradation (65a). Furthermore, mutations in Ser-32 and Ser-36 do not influence the stability of free IκBα (65b). Thus, it appears that the mechanism of signal-independent degradation of free IκBα is different in that it is dependent on the PEST sequence and not on N-terminal modification.

Parallel with *Drosophila* cactus protein. Dorsal, the *Drosophila* homolog of mammalian NF-κB/Rel proteins, and cactus, the homolog of IκBα, are key players in dorsoventral patterning of embryos (24). Recently it was reported that if the PEST sequence in cactus is deleted, it still can undergo signal-induced degradation (14). Additionally, in unstimulated dorsal mutant embryos, cactusΔPEST is more stable than full-length cactus. Interestingly, the cactusΔPEST mutant is able to rescue a lethal upstream gain-of-function mutation in the signal pathway. Thus, even though cactusΔPEST is degraded by signal, its stability as a free protein still has an impact on the overall development of the embryo, during which amounts of stimuli are more moderate than those used experimentally. Consequently, it appears that two separate systems for cactus degradation may exist: (i) signal dependent, association independent, PEST independent and (ii) signal independent, association dependent, PEST dependent. Thus, there is substantial degree of evolutionary conservation in the overall mechanisms of degradation for bound and free IκBα.

Are there common amino acid sequence motifs that participate in ubiquitin-proteasome-mediated degradation of proteins? A "destruction box" sequence has been shown to be required for ubiquitin-mediated degradation of mitotic cyclins (27, 39). In contrast, a region containing PEST sequences and multiple phosphorylation sites is required for degradation of CLN3 during cell cycle (69). On the other hand, degradation of the transcription factor MATα2 requires two different regions of the protein, which are unique to this protein (32). Thus, there is a considerable variation in requirements for degradation of proteins by the ubiquitin-proteasome system. The IκBα protein, crucial to regulation of NF-κB/Rel protein, has evolved multiple mechanisms of degradation depending on the need and whether it is bound or free. Understanding the precise molecular mechanism of degradation of IκBα protein will

be beneficial in designing drugs to prevent inflammation, septicemia, and a host of diseases dependent on the functioning of the immune system.

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