The PEST Domain of IκBα Is Necessary and Sufficient for in Vitro Degradation by μ-Calpain

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Polypeptide sequences enriched in proline (P), glutamate (E), serine (S), and threonine (T), dubbed PEST domains, are proposed to expedite the degradation of proteins. The proteolysis of one PEST-containing protein, IκBα, is requisite to the activation of the transcription factor NF-κB. Two mechanisms of IκBα degradation in vivo have been described, one well characterized through the ubiquitin-proteasome pathway, and another less characterized through calpain. In this report, a mutational analysis was done to identify any regions of IκBα that facilitate its recognition and proteolysis by calpain in vitro. These studies revealed that the PEST sequence of IκBα is critical for its calpain-dependent degradation. Furthermore, the IκBα-PEST domain binds to the calmodulin-like domain of the large subunit of μ-calpain (μCaMLD). Transfer of the IκBα-PEST domain to a protein incapable of either binding to or being degraded by μ-calpain allowed for the interaction of the chimeric protein with μCaMLD and resulted in its susceptibility to calpain proteolysis. Moreover, the μCaMLD of calpain acts as a competitive inhibitor of calpain-dependent IκBα degradation. Our data demonstrate that the IκBα-PEST sequence acts as a modular domain to promote the physical association with and subsequent degradation by μ-calpain and suggest a functional role for PEST sequences in other proteins as potential calpain-targeting units.

Underlying many of the complex cellular signaling networks are numerous posttranslational alterations of protein structure. Modifications of proteins can include phosphorylation, methylation, acetylation, proteolytic processing, and in some instances degradation of target molecules. A role for the latter has become clearer as discrete protein domains have been described that mark proteins for proteolysis, such as the KFERQ sequence (1), cyclin destruction boxes (2), and regions rich in proline (P), glutamic/aspartic acid (E), serine (S), and threonine (T), or PEST sequences (3). Two cytosolic proteases, the ATP-dependent proteasome and the calcium-dependent calpain, appear to be responsible for the majority of nonlysosomal targeted proteolysis (4). An example of inducible protein degradation is seen during the activation of the transcription factor NF-κB (5). Under nonstimulated conditions, NF-κB is partitioned to the cytoplasm through association with a member of the IκB family of inhibitory proteins, most notably IκBα (6). As IκBα binds to dimeric NF-κB complexes, it is able to mask the nuclear localization sequence present on NF-κB and thereby achieve cytoplasmic sequestration of the complex (7, 8). Activation of NF-κB, therefore, is typically preceded by the proteolytic inactivation of the IκB inhibitory protein (5). This has been shown to occur in response to many signals, which act to positively regulate an IκB kinase complex leading to site-specific phosphorylation of IκBα (9–12). This in turn targets the molecule to a ubiquitin kinase enzyme and subsequent degradation through the ubiquitin-proteasome proteolytic pathway (13, 14).

Although IκBα degradation is attributed primarily to the ubiquitin-proteasome pathway, there have recently been a number of alternate proteolytic mechanisms described for IκBα, including some which specifically implicate isoforms of calpain as the direct IκBα protease (15–18). A recent study suggests that loss of calpain 3 activity results in an accumulation of IκBα leading to an increased sensitivity to apoptosis, which contributes to the limb-girdle muscular dystrophy type 2A phenotype (15). Chen et al. (16) showed that IκBα is degraded in a calpain-dependent manner following treatment of the mouse macrophage cell line RAW 264.7 with the toxic particulate silica. Zhang et al. (17) showed that hypoxic conditions activated calpain activity in endothelial cells, resulting in a proteolysis of IκBα that was sensitive to the calpain inhibitor E-64d. Furthermore, Brasier and colleagues (18) demonstrated that treatment of HepG2 liver cells with the cytokine tumor necrosis factor-α resulted in the degradation of IκBα through the ubiquitin-proteasome pathway as well as through a parallel pathway dependent on calpain activity. We have also recently demonstrated a novel degradation pathway of IκBα in murine B cells that is independent of proteasome activity yet dependent on intracellular calcium and is associated with the constitutive activity of NF-κB in these cells (19). The sensitivity to cysteine-protease and calpain inhibitors as well as the calcium requirement of this IκBα proteolysis implicate a possible involvement of calpain. Together, these studies suggest that the degradation of IκBα required for NF-κB signaling is not solely dependent on the 26S proteasome but can also occur through calpain under certain conditions.

The calpain family of proteolytic enzymes is comprised of both ubiquitous and tissue-specific isoforms of calcium-dependent thiol proteases (20, 21). The ubiquitous μ- and m-calpains share structural similarity yet differ markedly in their requirements for calcium. In vitro, μ-calpain is active at calcium concentrations between 5 and 50 μM, whereas m-calpain is active at concentrations of 0.2–1.0 mM (22). Although μ-calpain re-

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quires far less calcium than m-calpain for its half-maximal activation in vitro, intracellular calcium concentrations are nevertheless orders of magnitude lower (0.1–0.4 μM). This poses an enigma surrounding the in vivo activation of calpains and has led to several proposed models of calpain regulation (22). These include interaction of calpain with membrane phospholipids, an autolytic self-activation, and its regulation by endogenous “activator” protein. Although their activation in cells remains unclear, several physiologic roles for the calpains have been suggested spanning such processes as cell-cycle regulation, apoptosis, and long term potentiation (20). Calpains are heterodimers consisting of a large 80-kDa subunit and a smaller 30-kDa subunit. The large subunit contains the catalytic function of the enzyme as well as a calmodulin-like domain. In addition, it contains a 30-kDa subunit. The large subunit contains the catalytic function of the enzyme as well as a calmodulin-like domain (CaMLD), so named for its high sequence homology to other EF-hand containing proteins such as calmodulin (20). Several known substrates of μ- and m-calpain have been identified and many more proposed (23), and yet structural determinants of calpain substrates remain ill-defined. Interestingly, several calpain substrates contain either calmodulin-binding domains or PEST domains (24). The latter have been associated with proteolysis, because proteins containing high PEST scores often undergo rapid degradation in vivo (3).

Ikβα contains a C-terminal PEST sequence and is reported to be inducibly degraded by calpains in vivo. Therefore, to better understand the mechanism allowing Ikβα to be degraded by calpain, and more specifically a possible role for the Ikβα-PEST domain in calpain-dependent proteolysis, we analyzed the Ikβα structural requirements for this reaction. Here, we report the fundamental role of the Ikβα-PEST sequence in determining substrate susceptibility to calpain in vitro. Our data suggest a mechanism for calpain-specific proteolysis of Ikβα in which first the PEST domain of Ikβα binds in a calcium-dependent manner to the CaMLD of the large subunit of calpain, followed by N-terminal cleavage of Ikβα and further proteolysis.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Constructs**—Murine Ikβα (pBS-mIkβα) and bacterial chloramphenicol acetyltransferase (CAT (pBS-CAT)) were cloned into pBlueScript (Stratagene). MutF and S32A/S36A Ikβα was generated by polymerase chain reaction (5′-AACTGGAGACAGCGCTACATGCCGCTAGCTGGGGGAAAGG-3′, forward, and 5′-CTCGTGGTGGATACGACG-3′, reverse) and cloned into BamHI/BclI of pBS-mIkβα. A 39-amino-acid C-terminal truncation of Ikβα has been described (26). CAT-PEST was generated by cutting Ikβα at Sau961, then ligating the blunt 3′ end to the 3′ CAT DNA at a created SmaI site immediately upstream of the CAT stop codon. N-CAT and N-CAT-PEST were formed by fusing the N-terminal 66 amino acids of Ikβα to either CAT or CAT-PEST, respectively, through two-step polymerase chain reaction. For N-CAT the primers used to amplify Ikβα 1–66 were (i) 5′-GAACTGGAGACAGCGCTACATGCCGCTAGCTGGGGGAAAGG-3′, forward, and (ii) 5′-AGTGATTTTTTTCTCC-CAGGGTCGGCAGCGCCGCGG-3′, reverse, and cloned into BamHI/BclI of pBS-mIkβα. A 39-amino-acid C-terminal truncation of Ikβα has been described (26). CAT-PEST was generated by cutting Ikβα at Sau961, then ligating the blunt 3′ end to the 3′ CAT DNA at a created SmaI site immediately upstream of the CAT stop codon. N-CAT and N-CAT-PEST were formed by fusing the N-terminal 66 amino acids of Ikβα to either CAT or CAT-PEST, respectively, through two-step polymerase chain reaction. For N-CAT the primers used to amplify Ikβα 1–66 were (i) 5′-GAACTGGAGACAGCGCTACATGCCGCTAGCTGGGGGAAAGG-3′, forward, and (ii) 5′-AGTGATTTTTTTCTCC-CAGGGTCGGCAGCGCCGCGG-3′, reverse, and cloned into CAT (or CAT-PEST) (iii) 5′-GCCGCGGACAGCGCTACATGCCGCTAGCTGGGGGAAAGG-3′, forward, and (iv) 5′-GCCGCGGCGCGCGCTACATGCCGCTAGCTGGGGGAAAGG-3′, reverse. These products were amplified together with (i) and (iv) and the polymerase chain reaction product inserted into pBS-CAT or pBS-CAT-PEST at XbaI/MscI. Glutathione S-transferase (GST-μCaMLD was described previously (27)). Integrity of all constructs was confirmed by direct nucleotide sequencing.

**Protein and Purification**—Calpain I (μ-calpain) purified from porcine erythrocytes was purchased (Calbiochem). All [35S]methionine-labeled proteins were transcribed (T3 RNA polymerase) and translated over 2 h at 30 °C in TNT rabbit reticulocyte lysate or TNT wheat germ extract where specified, using 1 μg of pBS constructs per 50 μl of reaction volume according to the manufacturer’s recommendation (Promega). GST and GST-μCaMLD were purified from exponentially growing Escherichia coli (strain BL-21) 2 h following induction with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside. Briefly, cells pellets were resuspended in ice-cold phosphate-buffered saline with protease inhibitors, lysed by sonication, and brought to 1% Triton X-100. The lysate was cleared at 10,000 × g for 5 min and 50% slurry glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) were added to supernatant and mixed gently for 30 min. Beads were washed 4 times in ice-cold phosphate-buffered saline, and purity of the protein was confirmed by SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie staining.

**In Vitro Degradation Reactions**—All reaction samples included 0.5–1.0 μl of [35S]methionine-labeled substrate protein (approximately 2 ng, see Fig. 1B), 750 μM CaCl₂, the indicated concentration of μ-calpain, and were incubated in calpain reaction buffer (30 mM Tris-HCl (pH 7.5) and 1.5 mM dithiothreitol). Reactions were brought to a final volume of 10 μl and following incubation at 30 °C for 15 min were terminated on ice by addition of 2× SDS sample buffer. For competition experiments, GST proteins coupled to Sepharose (as described below) were rinsed 4 times with calpain reaction buffer and 10 μl added to the reaction mixture for a final reaction volume of 20 μl.

**Pull-down Assays**—Association reactions were performed in the presence of 10 mg/ml E. coli protein extract in 1× binding buffer (50 mM KPi (pH 7.5), 150 mM KCl, 10% (v/v) glycerol, and 0.1 mg/ml apotinin A (Sigma)). For each sample 1 μl of [35S]methionine-labeled protein was incubated with 200 μl of E. coli extract on ice for 15 min, and cleared at 14,000 rpm for 15 min. 20 μl of GST or GST-μCaMLD bound to Sepharose beads (1:1 slurry) was added to this, and the final volume brought up to 0.5 ml with E. coli protein extract. Reactions were tumbled at 4 °C for 2 h, washed 5 times in ice-cold 1× binding buffer, boiled in 1× SDS sample buffer, and separated by SDS-PAGE.

**Immunoprecipitations and Immunoblotting**—Antibodies used were rabbit polyclonal raised against either the N-terminal 56 amino acids of murine Ikβα conjugated to GST (5432 antibody, see Ref. 28) or the C-terminal 21 amino acids of C21 antibody (N. Hunter, Nycomed USA, Inc.). Protein A-Sepharose and the appropriate antibodies were added to each reaction. IP buffer and protocol have been described previously (19). All immunoblotting was done with the C21 antibody as described previously (19).

**In Vitro Phosphorylation Reaction**—In vitro phosphorylation of Ikβα translated in rabbit reticulocyte lysate was performed according to Hunter et al. (29). Briefly, following translation the extract was passed through G-50 SephadeX (Amersham Pharmacia Biotech) equilibrated with 10 mM NaCl, 25 mM KCl, 1 mM MgCl₂, 10 mM Tris (pH 7.5), 0.25 mM dithiothreitol, 0.1 mg/ml bovine serum albumin. [γ-32P]ATP was added, and the reaction incubated at 30 °C for 60 min. Phosphatase inhibitors were then added to all but one wild-type Ikβα reaction, and the remaining [γ-32P]ATP removed by G-50 chromatography. The Ikβα sample lacking phosphatase inhibitors was incubated with 10 units of calf intestinal alkaline phosphatase (Roche Molecular Biochemicals) at 37 °C for 30 min. All Ikβα proteins were then immunoprecipitated with N-terminal-specific 5432 antibody and transferred to Immobilon-P following SDS-PAGE. Western blotting was done to confirm equal amounts of in vitro translated proteins, and phosphoproteins were visualized by autoradiography.

**RESULTS**

Ikβα Is Degraded by μ-Calpain in Vitro—To study the substrate characteristics of Ikβα for calpain it was important to first determine the calpain-specific cleavage and/or degradation of Ikβα in vitro. We analyzed the purity of commercially available μ-calpain extracted from porcine erythrocytes by SDS-PAGE. A Coomassie Blue stain of the purified calpain used (Fig. 1A) demonstrates that the major band migrates above the 68-kDa marker, representative of the large 80-kDa subunit. The small subunit of calpain, migrating at ~29 kDa, is also visible. Composition among different lots of enzyme differed (not shown). In addition, the sensitivity of Ikβα to degradation among the different μ-calpain lots also varied (not shown). The amount of in vitro translated Ikβα substrate added to reaction mixtures is estimated to be approximately 2
Because the proteasome is abundant in cell extract and known to degrade IxBα, we tested whether lactacystin, which specifically inhibits the proteasome by modifying the active site N-terminal threonine residue of the catalytic β subunit (31), could block the degradation of IxBα under these conditions. As shown in Fig. 1D, we found that a concentration of lactacystin as high as 100 μM had no effect on the reaction whereas calpeptin, a synthetic calpain inhibitor, showed a dose-dependent inhibition of IxBα proteolysis. These data indicate that IxBα translated in either a mammalian or a plant cell-free system is degraded by calpain in vitro.

The C-terminal PEST Domain of IxBα Facilitates Its in Vitro Degradation by Calpain—IxBα can be divided into three general domains. These include (i) an N-terminal domain that contains amino acid constituents required for the inducible degradation of IxBα (32–34); (ii) an internal region made up of six repeats of a 30–34 amino acid ankyrin-like domain that facilitates association with NF-κB (35, 36); and (iii) a C-terminal acidic domain that includes a PEST sequence and appears to be involved in regulating some cases of IxBα protein turnover (26, 37–39), as well as the inhibition of DNA binding by NF-κB (40, 41). We questioned whether either the N-terminal signal responsive domain or the C-terminal PEST domain of IxBα are determinants for the efficiency of in vitro degradation by calpain. This was tested through an analysis of calpain-mediated proteolysis using either N-terminally or C-terminally truncated IxBα proteins in comparison to the full-length IxBα protein as substrates. [35S]methionine-labeled IxBα proteins lacking either amino acids 1–36 (IxBΔN) or amino acids 278–314 (IxBΔC) were incubated with increasing concentrations of μ-calpain, terminated, and separated by SDS-PAGE (Fig. 2A). At a calpain concentration of 100 nM the wild-type IxBα protein was almost completely degraded within 15 min (Fig. 2B). The IxBΔN mutant was proteolyzed in a similar fashion. Proteolytic breakdown of IxBΔC, however, was reduced in comparison to full-length IxBα at equal concentrations of calpain. Following incubation with 100 nM calpain the majority of the input protein still remained. These data suggest that a sequence, or sequences, within the IxBα C-terminal domain promotes the in vitro degradation of IxBα by μ-calpain.

As a more direct test of the contributing role of the C-terminal PEST domain to IxBα as a calpain substrate, we examined the degradation profile of an IxBα mutant in which the PEST score has been lowered. MutF contains five alanine substitutions for serine and threonine residues within the PEST domain (25), lowering the PEST score of the C terminus from +4.6 to −0.7 (determined using the PEST-FIND algorithm developed by Rogers et al. (3)). When MutF was incubated with increasing calpain concentrations we detected a significant decline in proteolysis relative to that of the wild-type protein. At the highest calpain concentration tested MutF was only degraded to ~60% of its original amount, which represents a reduction in the proteolytic efficiency of calpain in vitro. MutF can bind to NF-κB when expressed in cells and is sensitive to the proteasome-dependent degradation pathway, and therefore gross structural alterations in the protein caused by amino acid substitution that result in proteolytic insensitivity are unlikely (25).

However, the amino acid replacements in MutF disrupt the basal phosphorylation of IxBα by casein kinase II (25, 42). Thus, we compared the phosphorylation status of the full-length protein to that of MutF following translation in rabbit reticulocyte lysate. Additionally, a mutant of the inducible phosphorylation sites, serines 32 and 36 within the N terminus of IxBα (32–34), was examined. Following depletion of cold ATP from the reaction mixture by G-50 chromatography,
[\gamma^{32}P]ATP was added to the translation reaction mixtures, and IkBα immunoprecipitated following the reaction (29). Fig. 3A shows that IkBα was phosphorylated in the reticulocyte lysate, and treatment with calf intestinal alkaline phosphatase (CIP) reduced the incorporated radiolabeled phosphate. Replacing serines 32 and 36 with alanine residues had no effect on the reduced the incorporated radiolabeled phosphate. Replacing and treatment with calf intestinal alkaline phosphatase (CIP) substrate of calpain and treatment with calf intestinal alkaline phosphatase (CIP) and then subjected to calpain proteolysis in both the presence and absence of EGTA. CIP-treated IkBα was less sensitive to calpain-specific degradation of the IkBα, and that the affinity of the protein is indeed necessary for association of the protein with calpain. Furthermore, IkBα that had been pretreated with CIP was less sensitive to calpain-specific degradation than untreated IkBα over time (Fig. 3C). These findings suggest that the integrity and/or posttranslational modifications of the IkBα PEST sequence can alter the efficacy of calpain to degrade IkBα in vitro.

The PEST Domain of IkBα Associates with the Calmodulin-like Domain of the Large Subunit of μ-Calpain—Since their first description in 1986, it has been speculated that PEST domains are able to increase a protein’s turnover rate through association with calpain (3). However, to our knowledge, neither calpain nor any other protease has been observed to directly interact with a protein’s PEST domain. The large subunit of μ-calpain contains a calmodulin-like domain (µCaMLD) that has been shown to mediate a calcium-dependent interaction with the endogenous calpain inhibitor calpastatin (27, 43). Thus, to test whether µCaMLD directly associates with IkBα, possibly through the IkBα-PEST domain, we used a fusion protein consisting of GST and the µCaMLD (GST-µCaMLD) and tested for its association with IkBα and deletion mutant proteins in a GST pull-down assay. A representative gel is shown in Fig. 4A. Following incubation, GST-µCaMLD, but not GST, was able to bind to full-length IkBα in the presence of calcium but only weakly when calcium was not added to the binding buffer. Alternatively, IkBα was dephosphorylated by CIP before the association reaction. The binding of IkBα to GST-µCaMLD was also weak, similar to that seen for MutF (Fig. 4B). These findings are consistent with and correlate well with the inability of IkBα to be degraded by µ-calpain and the reduced ability of MutF or CIP-treated IkBα to be degraded by µ-calpain (Figs. 2 and 3). These results suggest that the PEST-containing C-terminal domain of IkBα facilitates the association of IkBα with the µCaMLD of calpain in vitro and that the affinity of the protein substrate for µCaMLD affects its efficiency of degradation by calpain in vitro.

IκBα-PEST Acts as a Modular Domain to Allow Association with µCaMLD to an Otherwise Nonbinding Protein—Data from the GST pull-down experiments demonstrated that the PEST domain of IkBα is indeed necessary for association of the protein with µCaMLD. To ascertain whether the PEST domain itself is sufficient for binding the µCaMLD, we generated chimeric proteins in which bacterial CAT was fused to either N- or
C-terminal portions of IκBα. The N-terminal 66 amino acids of IκBα were fused to the N terminus of CAT to create N-CAT, whereas the C-terminal 54 amino acids of IκBα, inclusive of the PEST domain, were fused to the C terminus of CAT to create CAT-PEST (Fig. 5A). Following translation and incorporation of [35S]methionine in vitro, these proteins were incubated with either GST or GST-μCaMLD in both the presence and absence of added calcium, as previously for the IκBα proteins. CAT-PEST showed a strong association with GST-μCaMLD, which was favored by the addition of exogenous calcium to the binding reaction (Fig. 5A). However, neither CAT nor N-CAT were detectable in the fraction pulled down by GST-μCaMLD even on exposures up to 10 times longer than that required to visualize CAT-PEST. Immunoprecipitations demonstrated that CAT-PEST, but not CAT, is phosphorylated at similar levels as the IκBα protein during in vitro translation in rabbit reticulocyte lysate, consistent with the phosphorylation of a residue(s) within the IκBα-PEST domain (data not shown). Strikingly, these data show that the IκBα-PEST domain acts autonomously to bind the CaMLD of μ-caspase and that this association is transferable to a normally nonbinding protein.

As mentioned, the CAT-PEST fusion protein was pulled down by GST-μCaMLD even in the absence of added calcium, though slightly less efficiently than when calcium was added.

This interaction could indicate that the protein-protein association is not completely calcium-dependent. Alternatively, contaminating calcium ions co-purified with GST-μCaMLD from bacterial lysate or were provided from rabbit reticulocyte lysate and facilitated the interaction. When residual calcium ions were eliminated by the addition of EGTA to the binding buffer we observed that CAT-PEST no longer associated with GST-μCaMLD (Fig. 5B). Therefore, the binding of the IκBα-PEST sequence to GST-μCaMLD is calcium-dependent.

**Conferred Association with μCaMLD by IκBα-PEST Is Sufficient to Confer Susceptibility to Proteolysis to a Nonsubstrate Protein**—We have demonstrated that the PEST domain of IκBα is sufficient to allow the binding of a chimeric protein to μCaMLD in a calcium-dependent manner. Also, the association of IκBα with μCaMLD appears to be a requirement for its calpain-mediated degradation in vitro. This evidence suggests that by fusing the IκBα-PEST domain to a nonsubstrate protein we would be able to promote the association and subsequent proteolysis of the chimeric protein by calpain. Also, however, we have found using gradient gels and Tris-Tricine gel electrophoresis that calpain preferentially cleaves IκBα at one or both of two sites within the IκBα N terminus (data not shown). Consequently, in transferring the N-terminal 66 amino acids of IκBα to CAT we add to CAT one or more calpain cleavage sites. Therefore, to test whether the binding to calpain or the presence of a preferred calpain cleavage site(s) is sufficient for proteolysis, we analyzed the sensitivities to calpain-dependent degradation of the CAT protein and related CAT chimeric proteins.
calpain detects two intermediates that migrate at the predicted molecular mass for degradative products generated by cleavage at these two sites (Fig. 6B). Therefore, transfer of the IκBα N-terminal calpain cleavage sites alone to CAT is not sufficient to allow the fusion protein to be degraded by calpain, nor does it make the CAT-PEST chimera a better substrate for calpain. However, transfer of the IκBα-PEST domain to CAT does confer to the fusion protein sensitivity to calpain degradation in vitro.

We designed a competition experiment to test the model of calpain-dependent proteolysis in which association of IκBα to the μCaMLD of calpain precedes IκBα degradation. Increasing amounts of either GST (in molar excess over IκBα of up to 200-fold) or GST-μCaMLD (in molar excess over IκBα of up to 80-fold) were added to a reaction mixture containing [35S]methionine-labeled IκBα and calpain. Following incubation, the amount of IκBα substrate remaining was analyzed (Fig. 6C). Addition of GST-μCaMLD displayed a dose-dependent block of IκBα proteolysis, whereas similar amounts of GST added alone had no effect. To ensure that GST-μCaMLD did not directly inhibit calpain activity in these experiments, excess GST-μCaMLD was incubated with calpain before addition of IκBα substrate. The ability of the calpain protease to digest IκBα was not diminished (data not shown). Together these studies demonstrate that the IκBα-PEST sequence is both necessary and sufficient for association with the μCaMLD of calpain, which subsequently confers susceptibility to μ-calpain-dependent proteolysis in vitro.

DISCUSSION

Activation of the transcription factor NF-κB by a wide variety of inducing agents is preceded by the proteolytic inactivation of the inhibitory molecule IκBα. Because of its central role in NF-κB signaling, great effort has gone into delineating the events required to degrade IκBα through the ubiquitin-proteasome pathway. In addition to proteasome-dependent IκBα proteolysis, several groups have either directly or indirectly implicated calpain as an in vivo IκBα protease (15–19). However, the mechanism of IκBα degradation by calpain is uncertain. In this report we examined the structural determinants of IκBα required for degradation by calpain in vitro and demonstrate a functional role for the C-terminal PEST domain of IκBα as a necessary and sufficient calpain-targeting module. A necessary role for the PEST sequence is inferred because deletion of this region inhibits the degradation of IκBα by calpain, and mutation of residues within this domain reduces the efficiency of calpain-dependent IκBα proteolysis as well. Furthermore, the hierarchy of sensitivity to calpain degradation among the IκBα-PEST mutants is mirrored in their relative abilities to associate with the μCaMLD of the large calpain subunit, i.e. IκBα > MutF > IκBΔC. The finding that transfer of the IκBα-PEST domain to CAT yields a chimeric molecule that only then binds to and is a substrate for calpain indicates that this sequence is sufficient for calpain-dependent proteolysis.

Phosphorylation of some proteins within PEST or PEST-like sequences is necessary for their recognition by F-box or other proteins that serve to recruit the substrate to a ubiquitin-ligase complex, and degradation through the 26 proteasome ensues (44–48). Although it has long been proposed that by sequestering calcium, the PEST motif could form a calcium-dependent interaction with calpain that would place the catalytic site of calpain in close proximity to the PEST-containing substrate (3, 24) to our knowledge this is the first demonstration of direct association between a PEST sequence and calpain or any other protease.

We determined that the degradation of IκBα by calpain is preceded by cleavage at either one or both of two N-terminal

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**Fig. 6. Transfer of IκBα C terminus, not N terminus, to CAT confers susceptibility to calpain-mediated proteolysis in vitro.**

A. structures and calpain sensitivities of CAT and CAT-IκBα fusion proteins. The CAT protein is represented as a shaded box; N and PEST refer to IκBα amino acids 1–66, and 259–314, respectively. [35S]Methionine-labeled IκBα proteins were incubated with μ-calpain at the concentrations indicated for 15 min at 30 °C and terminated. Results are representative of two to three independent experiments. B. Western blot analysis of IκBα and N-CAT-PEST proteolysis by μ-calpain. Unlabeled IκBα or N-CAT-PEST proteins were incubated with 0, 5, 25, or 100 nM μ-calpain for 15 min at 30 °C and detected with IκBα C-terminal antibody. Proteolytic intermediates of IκBα and N-CAT-PEST are indicated by either filled or empty arrowheads, respectively. Molecular mass standards in kDa are indicated to the left. C. effect of GST-μCaMLD on calpain-dependent IκBα proteolysis. Either buffer alone (lane 10) or increasing amounts of GST (approximately 50, 100, 300, and 600 ng in lanes 2–5) and GST-μCaMLD (approximately 50, 100, 200, and 400 ng in lanes 6–9) bound to glutathione-Sepharose were added to reactions containing [35S]methionine-labeled IκBα and 100 nM calpain. After 10 min on ice, the reactions were incubated at 30 °C for 15 min and terminated samples were separated by SDS-PAGE followed by autoradiography (top panel). Four times the amounts of GST or GST-μCaMLD, which were added to each reaction, were separated by SDS-PAGE followed by Coomassie Blue (CB) staining and are shown in the bottom panel.

The CAT protein was not detectably proteolyzed by calpain even at the highest enzyme concentration tested (100 nM, Fig. 6A). On the other hand, the CAT-PEST fusion protein displayed a degradation profile similar to that of IκBα. N-CAT was only weakly susceptible to calpain proteolysis, but when the IκBα-PEST domain was fused to N-CAT (N-CAT-PEST) this chimeric protein was efficiently degraded by calpain in vitro. As expected, the 66 amino acids from IκBα that were fused to CAT appear to contain the two preferred calpain cleavage sites, because Western blotting with antibody raised against the IκBα C terminus following degradation of N-CAT-PEST by
have observed that in vitro, IκBβ is readily degraded by μ-calpain, suggesting potential calpain-mediated regulation of NF-κB complexes in association with IκB members other than IκBα. Additionally, it may be insightful to examine the ability of calpain substrates that lack high PEST scores to associate with the CaMLD. The findings in this report suggest that association through PEST sequences may be an indication of a protein’s sensitivity to calpain proteolysis, though one can imagine CaM binding domains or other uncharacterized motifs playing a similar role. Though the contribution of IκB-PEST to calpain-dependent IκBα proteolysis in vitro has not been investigated here, our demonstration of its calpain-targeting potential in vitro provides information on IκBα structure that can be used to examine systems where calpain is known or suspected to be activated.

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sites (data not shown). This is consistent with findings based both on μ-calpain and m-calpain (16, 18, 49). A strong preference for these sites by calpain is evident, because the N-CAT-PEST protein displays a similar pattern to IκBα as it is proteolyzed. The results suggest that one requisite role for calcium in calpain proteolysis may be proximity, i.e. the recruitment of the protein substrate to the enzyme. Although the 66 N-terminal residues of IκBα contain strong calpain cleavage sites, these alone were not sufficient to confer calpain susceptibility to CAT. However, when CAT was brought into association with calpain in the presence of calcium by means of the IκBα-PEST sequence, proteolysis ensued. Thus our data indicate that a calcium-dependent association, not catalysis, is the rate-limiting step in this reaction. Though it was not demonstrated, a similar role for the PEST sequences of the common cytokine receptor γ chain may exist, because this protein bound to the CaMLD of the calpain small subunit and was not degraded by calpain when the PEST sequences were removed (50).

Recently, evidence has accumulated, which suggests that the ubiquitin-proteasome proteolytic pathway may be responsible for degradation of many PEST-containing proteins (51). Indeed, recent reports demonstrate that the PEST domains of three different calpain substrates are dispensable for calpain proteolysis (52–54). In one instance it was shown that rather than the PEST domains, the calmodulin-binding domain of the calcium-dependent ATPase was critical for its degradation by calpain (52). A common theme among the vast majority of calpain substrates is that they contain either a PEST sequence, a CaM binding domain, or both. Though unlikely to apply to all calpain substrates, it is intriguing to think that in these proteins the PEST domains or CaM binding domains serve overlapping functions, i.e. by promoting the association of substrate with μCaMLD these protein motifs could act interchangeably as calpain-homing sequences.

Several groups have shown that the basal turnover of IκBα becomes retarded when the casein kinase II phosphorylation sites within the PEST sequence are deleted or replaced with alanine (25, 26, 39, 55, 56). Interestingly, our data indicate that phosphorylation of IκBα at casein kinase II sites within the PEST domain enhances both the association with and the degradation by calpain in vitro, consistent with the possibility that calpain activity, either alone or in addition to the proteosome (56), may be responsible for the basal degradation of IκBα under some in vitro conditions.

Furthermore, we show that the N-terminal signal responsive domain of IκBα does not affect calpain-dependent proteolysis. In keeping with this, we discovered that neither the induced phosphorylation of IκBα at serines 32 and 36, nor the substitution of alanine at these sites, alters the rate and pattern of IκBα degradation by calpain (data not shown). These data indicate that the S32A/S36A mutants, which have been used to identify the inducible phosphorylation-dependent NF-κB activation pathway, can be effectively degraded by calpain. Thus, the S32A/S36A IκBα protein will likely prove to be a useful reagent in distinguishing the two proteolytic mechanisms. Similarly, future investigations directed at determining the amino acid components and points of contact along the interface between IκBα-PEST and μCaMLD could be helpful to generate IκBα mutants specifically resistant to calpain-dependent proteolysis. Such studies may also help to specify the role of PEST motifs as general calpain-targeting sequences.

It will also be of interest to resolve which, if any, PEST sequences derived from other proteins, such as other IκB members, are able to interact with the CaMLDs of calpain. IκBβ has a C-terminal PEST sequence similar to IκBα, and IκBε has putative PEST sequences in its amino-terminal domain. We

2 S. D. Shumway and S. Miyamoto, unpublished results.
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