Domain Structure of Calpain: Mapping the Binding Site for Calpastatin†

Dorothy E. Croall* and Kevin S. McGrody‡

Department of Biochemistry, Microbiology, and Molecular Biology, University of Maine, Orono, Maine 04469-5735

Received July 5, 1994; Revised Manuscript Received August 25, 1994®

ABSTRACT: The peptide EKLGERDDTIPPEYRELLEKKTGV was synthesized to mimic the central consensus sequence of calpastatin, the specific, endogenous inhibitor of the calpains (EC 3.4.22.17). The peptide competitively inhibits hydrolysis of casein by either micro- or milli-calpain but does not affect the activity of other proteases. This inhibitory peptide was preferentially cross-linked to milli-calpain in the presence of calcium using the heterobifunctional cross-linking reagent *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester. Cross-linking of the peptide was blocked by calpastatin. The site of cross-linking for the peptide within milli-calpain was localized using random chemical cleavage of the enzyme−peptide complex at cysteine residues. Calpain fragments were identified as amino-terminal fragments through reactivity with a peptide-specific antiserum or as non-amino-terminal fragments through incorporation of ^{14}C from ^{14}CN. Analysis of the control and cross-linked fragments, from experiments using both milli-calpain and micro-calpain, maps the chemical cross-linking site to cysteine-497 and localizes the binding site for the calpastatin-like peptide to this highly conserved region of domain III of calpains catalytic subunit.

The calpains (EC 3.4.22.17) are ubiquitous, intracellular, nonlysosomal, calcium-dependent, cysteine-type proteases [for recent reviews, see Croall and DeMartino (1990, 1991), Mellgren and Murachi (1990), Suzuki and Ohno (1990), and Suzuki et al. (1989)]. There are two well-characterized calpains, micro(μ)-calpain and milli(m)-calpain. In mammalian tissues, each enzyme is a heterodimer composed of a distinct catalytic subunit (approximately 80 kDa) and an identical regulatory subunit (approximately 30 kDa) (Figure 1A). Each calpain is synthesized as a proenzyme that undergoes limited autoproteolytic processing of each subunit, which increases the enzymes' sensitivity to calcium and activates proteolysis of other substrates (Coolican et al., 1986; DeMartino et al., 1986; Inomata et al., 1988; Croall, 1989; Cottin et al., 1991; Croall et al., 1992). The amino acid sequences of the enzymes, as inferred from cDNAs (Mellgren & Murachi, 1990; Aoki et al., 1986; Imajoh et al., 1988; McClelland et al., 1989), reveal calmodulin-like domains (IV and IV′) within each subunit, but which of the eight putative EF-hand motifs actually bind Ca^{2+} to regulate enzyme activity is uncertain. The physiological functions and regulation of calpain are thought to be linked to calcium-regulated signalling pathways. These functions may include limited proteolysis of membrane cytoskeletal proteins (Beckerle et al., 1987; Hall & Bennett, 1987; Harris et al., 1988, 1989; Fox et al., 1991), transmembrane proteins (James et al., 1988; Wang et al., 1989; Giancotti et al., 1992), kinases and phosphatases (Adachi et al., 1989; Kishimoto et al., 1989, Croall & DeMartino, 1991; Frangioni et al., 1993), and calmodulin binding proteins (Wang et al., 1989; Choi et al., 1990; Brumley & Wallace, 1989). The degradation of some short-lived "PEST" proteins (Wang et al., 1989; Choi et al., 1990) and myofibrillar or cytoskeletal proteins (Cong et al., 1989; Croall & DeMartino, 1991; Litersky & Johnson, 1992) has also been proposed as a role for calpains.

One key to understanding calpain function and regulation *in vivo* is to determine how the endogenous proteinaceous inhibitor calpastatin (DeMartino & Croall, 1984; Cong et al., 1989; Mellgren & Murachi, 1990; Nishimura & Goll, 1991) interacts with calpain to regulate proteolysis. Calpastatin exists predominantly as a polypeptide of 713−718 residues that migrates anomalously as 120 kDa on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS−PAGE).[1] It inhibits 4 mol of calpain/mol of calpastatin. The inhibitory activity is heat-stable and can be attributed to a relatively short sequence repeated within calpastatin (Figure 1B). The functional domain was defined by the activity of multiple recombinant and synthetic peptides (Maki et al., 1988, 1989; Kawasaki et al., 1989; Mellgren & Murachi, 1990). Calpastatin is present in all cells that contain calpains and only binds to calpains in the presence of calcium (DeMartino & Croall, 1984: Cong et al., 1989; Nishimura & Goll, 1991). However, it is not clear what site within calpain is recognized for binding by calpastatin. Our approach to define the calpain−calpastatin interaction has been to exploit the well-characterized, small functional domain of calpastatin and its calcium-dependent binding to native calpain. In this report, we describe a synthetic peptide that mimics the inhibitory regions of calpastatin. Chemical modification of the peptide allowed covalent cross-linking to milli-calpain, and cross-linking occurred preferentially in the presence of calcium. Analysis of the cross-linked

† Supported by grants from the National Science Foundation (DCB 90-96188 and MCB 9319602) to D.E.C. and by a summer fellowship to K.S.M. from the Maine Affiliate of the American Heart Association.

* Address correspondence to this author. Phone: 207-581-2829. Fax: 207-581-2801. e-mail: croall@maine.maine.edu.

‡ Current address: M.D./Ph.D. Program, University of Rochester Medical Center, Rochester, NY 14642.

® Abstract published in *Advance ACS Abstracts,* October 1, 1994.

[1] Abbreviations: TIPPEYRin, synthetic peptide EKLGERDDTIPPEYRELLEKKTGV; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; MOPS, 3-(*N*-morpholino)propanesulfonic acid; DTNB dithiobis(2-nitrobenzoic acid); SDS−PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

0006-2960/94/0433-13223$04.50/0

products provides identification of one site of interaction between calpastatin and the catalytic subunit of milli-calpain.

EXPERIMENTAL PROCEDURES

Purification of Calpain and Calpastatin. Milli-calpain and micro-calpain were purified from frozen bovine cardiac tissue essentially as described previously (Croall & DeMartino, 1984). The enyzmes are routinely stored in the presence of 1 mM dithiothreitol, 1 mM EGTA, 1 mM EDTA, and 0.05% NaN_3 at pH 7.5 at 4 °C. Calpastatin was also purified from bovine heart (DeMartino et al., 1988) and stored at −80 °C.

Modification of TIPPEYRin[1] with m-Maleimidobenzoyl-N-hydroxysuccinimide Ester (MBS). The TIPPEYRin peptide, EKLGERDDTIPPEYRELLEKKTGV, was synthesized (by Dr. C. A. Slaughter, Howard Hughes Medical Institute, Southwestern Medical Center, Dallas, TX) using solid-phase, tBoc chemistry in an Applied Biosystems Model 430A peptide synthesizer. The peptide [1 mM in 150 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.5] was mixed with freshly prepared MBS in dimethylformamide at a 1:30 molar ratio (peptide amino groups to MBS). After 30 min at 25 °C, the sample was centrifuged to remove any precipitate and the supernatant chromatographed on a 0.7 × 4 cm column of Sephadex G-15 to separate the modified peptide from unreacted MBS. The peak fractions containing peptide were pooled and mixed with calpain in the presence or absence of calcium for cross-linking. The protein concentration of the modified peptides was determined (Bradford, 1976). In some experiments, the extent of MBS modification of TIPPEYRin was measured by incubation of the modified TIPPEYRin with a known concentration of 2-mercaptoethanol and Ellman's assay for free thiols (Ellman, 1959). Results demonstrated approximately one MBS incorporated per TIPPEYRin (0.7−0.9 mol/mol $n = 7$).

Cross-Linking Reactions. Immediately prior to cross-linking, calpain storage buffer was exchanged (by dilution and Centricon 30 spin concentrators, Amicon) with 150 mM MOPS, pH 7.5, to dilute dithiothreitol by at least 1000-fold. In the cross-linking reaction, the modified peptide concentration was 10−50 μM, and calpain was at 0.5−3 μM. Cross-linking in the presence of calcium was at a final concentration of 7.5 mM calcium and 0.375 mM leupeptin. Cross-linking reactions in the absence of calcium contained 4 mM EGTA. Incubation times, at 25 °C, are given in the figure legends. Cross-linking was terminated by addition of excess dithiothreitol (to both reactions) and EGTA to chelate calcium. Samples were incubated 1 h under reducing conditions prior to further analysis.

Random Cleavage of Calpain at Cysteine Residues. Reduced calpain, before or after cross-linking, was incubated with dithiobis(2-nitrobenzoic acid) (DTNB, final concentration 15 mM) for 15 min at room temperature. Samples were diluted 1:1 with glacial acetic acid, dialyzed against 10% acetic acid, and then lyophilized. Samples were resolubilized in 0.1 M sodium borate, 0.5 M glycylglycine, pH 9.0, and 8 M urea (Boehringer) for cyanoylation and cleavage at modified cysteines. A final concentration of 1 mM sodium cyanide (or [^{14}C]cyanide, Sigma or ICN) was added, and samples were incubated for 60 min at 40 °C. Reactions were terminated by the addition of dithiothreitol to 0.1 M, and then heating in SDS−sample buffer for electrophoresis. These methods are essentially as described by Nefsky and Bretscher (1989).

Miscellaneous Procedures. Proteolytic activity was measured using ^{14}C-methylated casein as substrate as previously described (DeMartino et al., 1986; Croall & DeMartino, 1984). Electrophoresis of Cys cleavage fragments was performed using a Tris−Tricine buffer system (Schagger & von Jagow, 1987). Colloidal Coomassie blue G250 (Neuhoff et al., 1988) was used to stain gels prior to treatment with ENLIGHTNING (New England Nuclear, NEN), drying, and fluorography (Fuji RX) for ^{14}C-labeled samples. Iodination of TIPPEYRin was carried out with Iodo-beads (Pierce) and Na^{125}I (NEN).

RESULTS

Inhibition of Calpain by the TIPPEYRin Peptide. Synthetic and recombinant peptides representing each specific domain of calpastatin have previously been shown to inhibit milli- and micro-calpains (Emori et al., 1988; Maki et al., 1988, 1989; Kawasaki et al., 1989). The TIPPEYRin sequence was selected as a hybrid of the most highly conserved region of the four functional domains of calpastatin (Figure 1B). The peptide most closely resembles domain IV of calpastatin (17/24 residues are identical) and domain I which are the two domains with highest affinity for calpain (Kawasaki et al., 1989). Because this peptide was not identical to any of those previously studied, we assayed each calpain at various concentrations of casein in the presence or absence of TIPPEYRin to (1) confirm the peptide's ability to inhibit calpain and (2) determine its apparent mechanism of inhibition. Results representative of these experiments are shown in Figure 2 and demonstrate competitive inhibition of each calpain by the peptide, using casein as the substrate. These results are similar to those reported for other calpastatin-related peptides (Maki et al., 1989). Half-maximal inhibition was achieved at 0.5−2 μM TIPPEYRin. Inhibition by TIPPEYRin was also specific for calpains. Neither papain nor α-chymotrypsin was inhibited by 20 or 50 μM TIPPEYRin.

Cross-Linking TIPPEYRin to Milli-Calpain. Calpastatin interacts with calpain only when calpain has bound calcium (DeMartino & Croall, 1984; DeMartino et al., 1988; Cong et al., 1989; Nishimura & Goll, 1991; Ma et al., 1993). Thus, the functional domain of calpastatin was expected to bind to calpain in a calcium-dependent interaction, and we predicted that efficient and specific cross-linking of the TIPPEYRin peptide to m-calpain would also be calcium-dependent. A two-step cross-linking procedure was selected to avoid cross-linking within or between the calpain subunits. Initial studies using a hydrophilic, zero-length cross-linker, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, in the presence of *N*-hydroxysuccinimide (Graberek & Gergely, 1990) to modify the peptide did not produce a cross-linked product (data not shown). The heterobifunctional reagent MBS (0.99 nM) was subsequently used to modify the TIPPEYRin peptide. The peptide contains an α-amino group and three lysine residues but has no cysteines and therefore can be modified selectively. Modification resulted in approximately one maleimide incorporated per TIPPEYRin (see Experimental Procedures). Inactivation of the maleimide with β-mercaptoethanol allowed assay of the modified peptide to confirm its ability to inhibit calpain activity. Inhibition was essentially as effective as the unmodified peptide ($n = 3$), demonstrating that the modified peptide still binds to calpain at the functional site of inhibition.

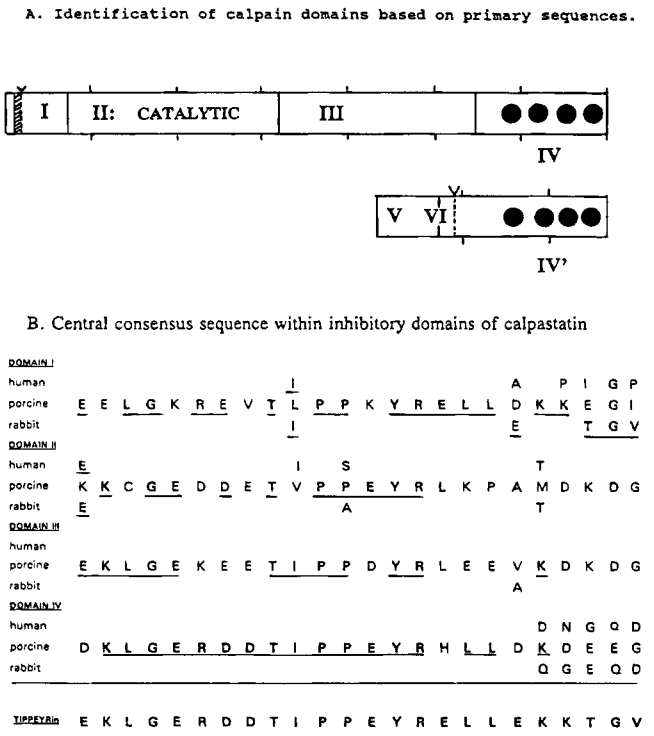

FIGURE 1: Organization of functional domains of calpains and calpastatin inferred from their amino acid sequences. (A) The two subunits of calpains are represented from their amino termini (left) to carboxy termini (right). Tick marks indicate 100 amino acids. Solid bars separate domains I, II, III, and IV of the 80 kDa subunits, and a V-dashed line indicates the autoproteolytic cleavage sites. The diagonally-lined region of the 80 kDa subunit depicts the location of the peptide used to raise antisera (Croall et al., 1992). The catalytic domain is designated as proposed by Suzuki (Suzuki & Ohno, 1990; Suzuki et al., 1987) based on homology to other cysteine proteinases. Domains IV and IV′ represent the calmodulin-like domains with closed circles indicating putative EF-hands. Domain V is the Gly-rich N-terminal region, and VI is a proline-rich hinge of the 26 kDa subunit. (B) Analysis of calpastatin from several mammalian sources identifies four functional domains (Mellgren & Murachi, 1990; Maki et al., 1988, 1989). The most highly conserved sequences in the central region of each domain are shown. The underlined, boldface residues are those present in the calpastatin-related peptide designated as TIPPEYRin. This 24-mer is 70.8% identical to the central consensus sequence of calpastatin domain 4 (residues 2−15, 17, 18, and 20). Residues 1, 16, 19, and 21−24 are identical to domain 1 from rabbit.

Although the sequence of the catalytic subunit of bovine m-calpain has not been determined, milli-calpains of known sequence (complete: human, rat; partial: rabbit, pig; Mellgren & Murachi, 1990; DeLuca et al., 1993; Sun et al., 1993) are approximately 94% identical. The bovine regulatory subunit sequence (McClelland & Hathaway, 1990) differs from human by only 4/268 residues (98.5% identical). The calpastatin binding site is expected to be highly conserved on the basis of calpastatin's conserved consensus sequence (human, pig, rabbit) and its cross-species effectiveness in inhibiting various calpains. Milli-calpain contains 15−16 Cys residues within the 80 kDa subunit including the catalytically active one. Nine Cys residues are conserved between all known calpain catalytic subunits and therefore are expected to be conserved in the bovine sequences. It was predicted that a cysteine would be close enough to the peptide binding site to allow cross-linking. Cross-linking to the catalytically active Cys, or to a Cys that is seven residues from the catalytic residue, might be possible since the peptide inhibits competitively. To unequivocally demonstrate the calcium-dependent formation of the cross-linked

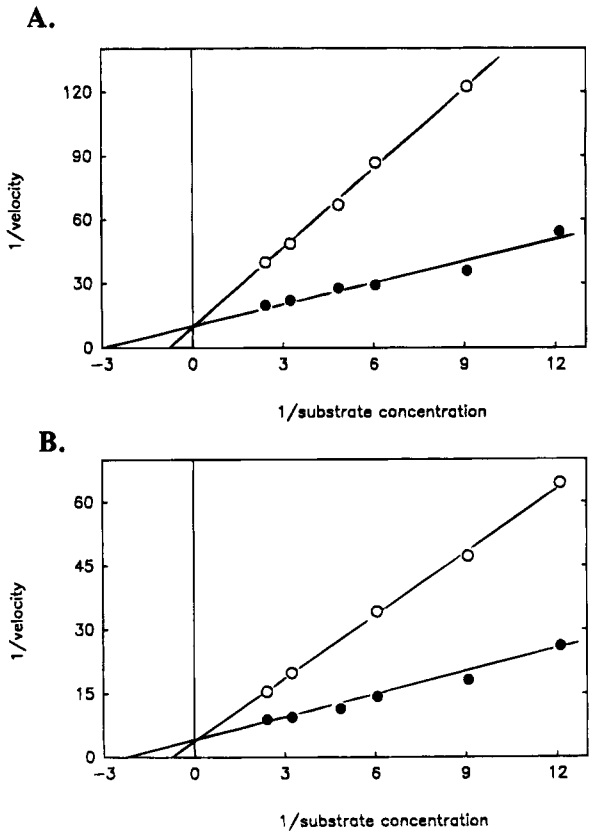

FIGURE 2: Lineweaver−Burk plots of calpain activity in the presence and absence of TIPPEYRin: Calpain activity was measured at various concentrations of ^{14}C-methylated casein in the absence (●) or in the presence of 5 μM TIPPEYRin peptide (○). Results are plotted as a double reciprocal of substrate concentration and velocity. Both substrate concentration and casein hydrolyzed were expressed relative to an arbitrary unit of radiolabeled casein. As the assay provides only relative, apparent rates of peptide bond hydrolysis, absolute units are not given. Results shown are from a single experiment for each enzyme and are representative of three such experiments for micro-calpain (panel A) and milli-calpain (panel B).

product, a time course for cross-linking was established using ^{125}I-TIPPEYRin. Results are shown in Figure 3A. There is some chemical reactivity of the MB-TIPPEYRin with the free thiols of calpain in the absence of Ca^{2+}, and with longer incubation times, more calcium-independent cross-linking occurs. The calcium-independent cross-linking of TIPPEY-Rin, however, appears to be randomly distributed between reactive thiols such that the rapid, site-specific cross-linking favored by the Ca^{2+}-dependent peptide−protein interaction in m-calpain is readily distinguishable. Importantly, when calpastatin is included in the cross-linking reactions containing calcium and ^{125}I-TIPPEYRin, cross-linking is blocked (Figure 3B). This clearly demonstrates that the TIPPEYRin binding site is within the site occupied by calpastatin.

Mapping the Site of Cross-Linking. (1) Random Chemical Cleavage at Cysteine Residues. Cleavage of calpain at Cys residues demonstrated reproducible fragments (Figure 4A,B,C) that were consistent with predicted locations of the conserved Cys residues. Amino-terminal fragments were identified by immunoblotting (Figure 4B) with an anti-peptide antiserum (Croall et al., 1992), allowing assignment of the Cys sites relative to this epitope (amino acids 4−23 of the human m-calpain sequence) as shown in Table 1. Both bovine m- and μ-calpains appear to have two additional Cys residues (approximately residues 225 and 475) relative to the human sequences on the basis of N-terminal fragments at 26 and

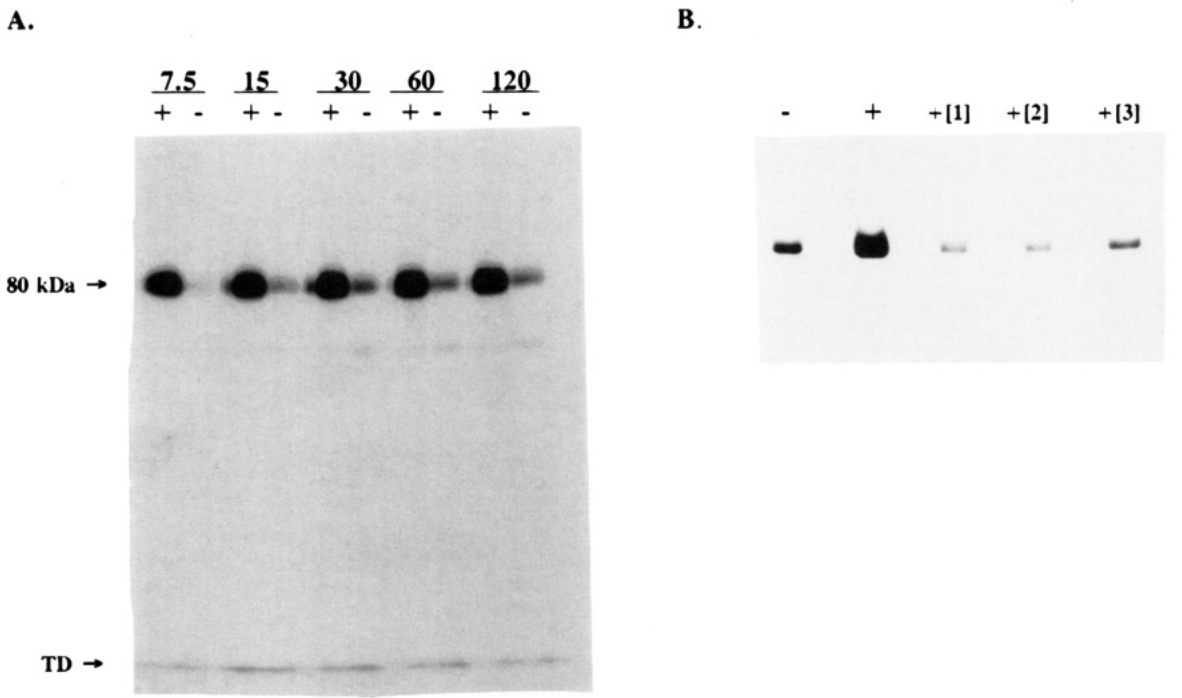

FIGURE 3: Calcium-dependence of cross-linking ^{125}I-TIPPEYRin to milli-calpain. Panel A: Radioiodinated TIPPEYRin was modified with MBS and then incubated with milli-calpain in the presence (+) or absence (−) of calcium as described under Experimental Procedures. At times shown (minutes), samples were treated with excess dithiothreitol and heated in SDS−sample buffer. The photo shows an autoradiograph from samples electrophoresed on a 10% acrylamide gel using Laemmli buffers (Laemmli, 1970). Migration of the stained 80 kDa protein band and the bromphenol blue tracking dye (TD) is marked. Panel B: Radioiodinated TIPPEYRin was modified with MBS and incubated with m-calpain (0.5 μM) in the absence (−) or presence (+, +[1], +[2], +[3]) of calcium for 30 min. Cross-linking reactions designated +[1], +[2], and +[3] also contained calpastatin at 0.6, 0.3, or 0.1 μM, respectively. The photo shows an autoradiograph of the cross-linked product from these reactions.

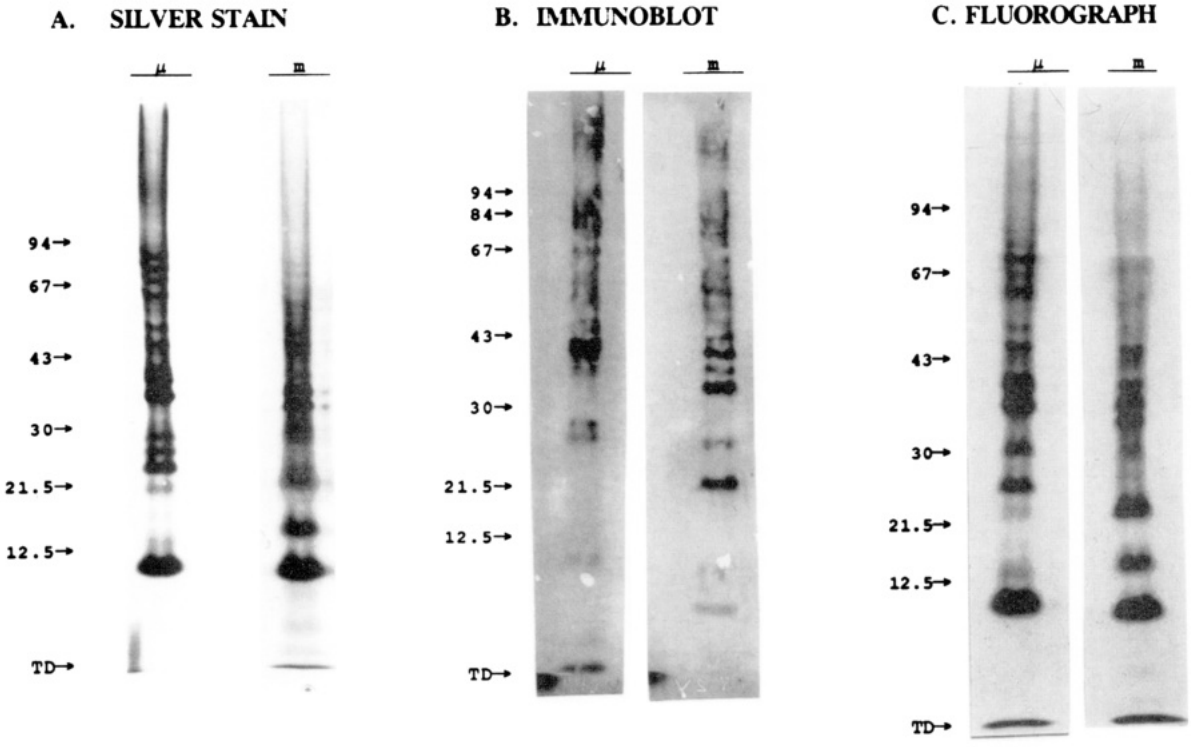

FIGURE 4: Random chemical cleavage of calpains at cysteine residues. Micro(μ)- or milli(m)-calpain was modified with DTNB and cleaved using ^{14}CN as described under Experimental Procedures. Protein fragments were displayed on 8% acrylamide gels and visualized by silver staining (panel A), by probing Western blots on nitrocellulose with N-terminal peptide-specific antiserum (panel B), or by fluorography (panel C). The molecular mass standards were phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa), and cytochrome *c* (12.5 kDa). TD indicates the tracking dye; panel B also shows the migration of the catalytic subunit of μ-calpain at 84 kDa. Aprotinin (6.5 kDa) was also used as a marker for some gels.

54 kDa. When [^{14}C]cyanide was used in the cleavage reaction, all non-amino-terminal fragments were radiolabeled and visualized by fluorography (Figure 4C). Non-amino-terminal fragments may either be C-terminal for molecules cleaved once or internal (neither N-terminal nor C-terminal) when the molecule is cleaved at more than one cysteine.

Table 1: Calpain Fragments Generated by DTNB−CN Cleavage and Resolved by Electrophoresis[a]

N-terminal (kDa)	est aa	theor Cys residues	^{14}C-labeled peptides (kDa)	est aa	est Cys residues	theor Cys residues
78	684	696				
75	660	665#	66	589	112	104#
73	640	640	60	526	175	190
64	560	577#	55	482	219	[ ]
58	509	497	45	395	306	300
54	475	[ ]	36	316	385	373#
46	405	404#	33	290	411	404#
42.4	372	373#	30.3	263	438	int.
38.5	338	340#	25	219	482	[ ]
34.2	300	300	22	193	508	497
27.5	241	239#	19	167	534	int.
25.6	225	[ ]	17	149	552	int.
22.2	195	190	16	140	561	577#
13	114	104#	11.5	101	600	int.
10.4	91	97#	7.9	68	633	640
7.3	64	81				

[a] The molecular mass determinations are based on averages of 3−5 independent experiments and gels. N-Terminal peptides were designated by reactivity with peptide-specific antiserum. ^{14}C-Labelled peptides were identified by fluorography. The theoretical Cys residues given are based on the published sequences of chicken, human, and rat calpains and the partial sequence of rabbit calpains (Mellgren & Murachi, 1990; DeLuca et al., 1993). The total number of fragments produced by random cleavage at n cys residues is $(n + 1)(n + 2)/2$ [see Nefsky and Bretscher (1989)]. For the 80 kDa catalytic subunit of human m-calpain where $n = 16$ cysteines, there are 153 possible peptides; of these, n will be N- or C-terminal (i.e., 16 for human m-calpain) peptides with the remainder being internal (int.). "#" denotes sites of Cys residues conserved among all known calpain 80 kDa subunits. [ ] designates apparent Cys residues specific to bovine calpains.

Assuming the simplest case of one cleavage site per molecule, the Cys pattern of the higher molecular weight fragments was consistent with conserved cysteines as summarized in Table 1.

(2) Cys Cleavage Fragments from Cross-Linked Products. Calpain from the cross-linking reactions was analyzed by immunoblotting and by fluorography of Cys-cleaved ^{14}C-labeled samples. Immunoblots with the N-terminal peptide-specific antiserum allowed mapping the site of cross-linking relative to the N-terminus in a strategy similar to that described by Sutoh (1982). An advantage of this approach is that cross-linking produces dominant, qualitative changes in peptide patterns. Molecules cleaved C-terminal to the site of cross-linking will contain the cross-linked peptide and therefore are not aligned with fragments produced in control reactions and are unique to the cross-linked sample. Any calpain that was not cross-linked to peptide and all fragments produced by cleavages N-terminal to the site of cross-linking will not contain the cross-linked peptide and will be identical in controls and cross-linked samples. (Variations in the yield of specific peptides may occur as a result of many factors, but these quantitative changes are not indicative of cross-linking, and thus not a factor in interpreting the data.) In addition to the cross-linking reaction in the absence of calcium, another control was also carried out for these experiments. A "mock" cross-linking reaction, consisting of m-calpain, calcium, and unmodified TIPPEYRin, was analyzed to ensure that no changes in calpain fragmentation resulted from the reversible interaction of calpain with calcium, or TIPPEYRin. Thus, our criteria for attributing band misalignment to specific cross-linking required that the calpain fragment, in the Ca^{2+}-dependent cross-linked sample, be distinct from both control fragment patterns. There were no consistent qualitative differences in the well-resolved, lower molecular mass immunoreactive fragments: N-terminal fragments less than or equal to 37 kDa ($n = 5$) (Figure 5A,B). Amino-terminal fragments in the higher molecular mass range, however, were never sufficiently resolved on the nitrocellulose to directly observe the crossover point for aligned and nonaligned bands when comparing the products from the two cross-linking reactions (i.e., plus or minus Ca^{2+}) and from the mock cross-linking control (plus calcium and unmodified TIPPEYRin). These data suggest that the peptide is cross-linked to a site at least 37 kDa from the N-terminal epitope.

The remaining fragments of the cross-linked samples were visualized by fluorography as shown in Figure 5C. Cys cleavage products smaller than 22 kDa are well aligned in all samples, but at, or above, this molecular mass, there are very distinct and unique fragments derived from samples cross-linked in the presence of Ca^{2+} (bands ▲ at 24.5 and 36 kDa). These distinct bands were also visible by silver staining (data not shown), suggesting that cross-linking was sufficiently quantitative for detecting cross-linked species. If we assume the simplest case, where these altered fragments result from a single cleavage of calpain, the data indicate that the cross-linking site is at least 22 kDa from the carboxyl end of the molecule.

(3) Differences between TIPPEYRin Cross-Linking to Milli- and Micro-Calpains. The calpastatin binding sites of the two calpain isoforms are predicted to be highly homologous on the basis of the overall homology of the enzymes and their shared susceptibility to inhibition by calpastatin. The conditions established for cross-linking ^{125}I-TIPPEYRin to milli-calpain also resulted in TIPPEYRin cross-linking to micro-calpain that was favored in the presence of calcium (Figure 6A). However, in contrast to the results with m-calpain, the pattern of Cys cleavage products generated from μ-calpain cross-linked in the presence or absence of calcium demonstrated no major differences. Analysis of the ^{14}C-labeled Cys cleavage products of cross-linked μ-calpain did not show distinct differences (Figure 6B). Cleavage patterns of the ^{125}I-TIPPEYRin-cross-linked products of μ-calpain also were indistinguishable from control (minus calcium) (Figure 6C). In contrast, Cys cleavage of m-calpain cross-linked to ^{125}I-TIPPEYRin in the presence of calcium revealed distinct labeled fragments, including major bands at 34 and 36−37 kDa (Figure 6C), consistent with our conclusions that the unique non-amino-terminal fragments observable by ^{14}C-labeling (Figure 5C) and also by silver staining (data not shown) did in fact contain the cross-linked inhibitory peptide. Attempts to immunopurify the ^{125}I-TIPPEYRin-cross-linked m-calpain fragments using antisera against either the amino-terminal epitope or an epitope in domain II (residues 282−301) were unsuccessful, but the negative results are consistent with a cross-linking site distant from these epitopes (data not shown). The contrasting results obtained for milli- and micro-calpain imply that the TIPPEY-Rin was not selectively cross-linked to any of the nine conserved cysteines shared by the two enzymes.

DISCUSSION

Calpastatin is a unique protease inhibitor. The protein contains 4 functional repeats, of about 140 amino acids, that are homologous to one another. Within each repeat there

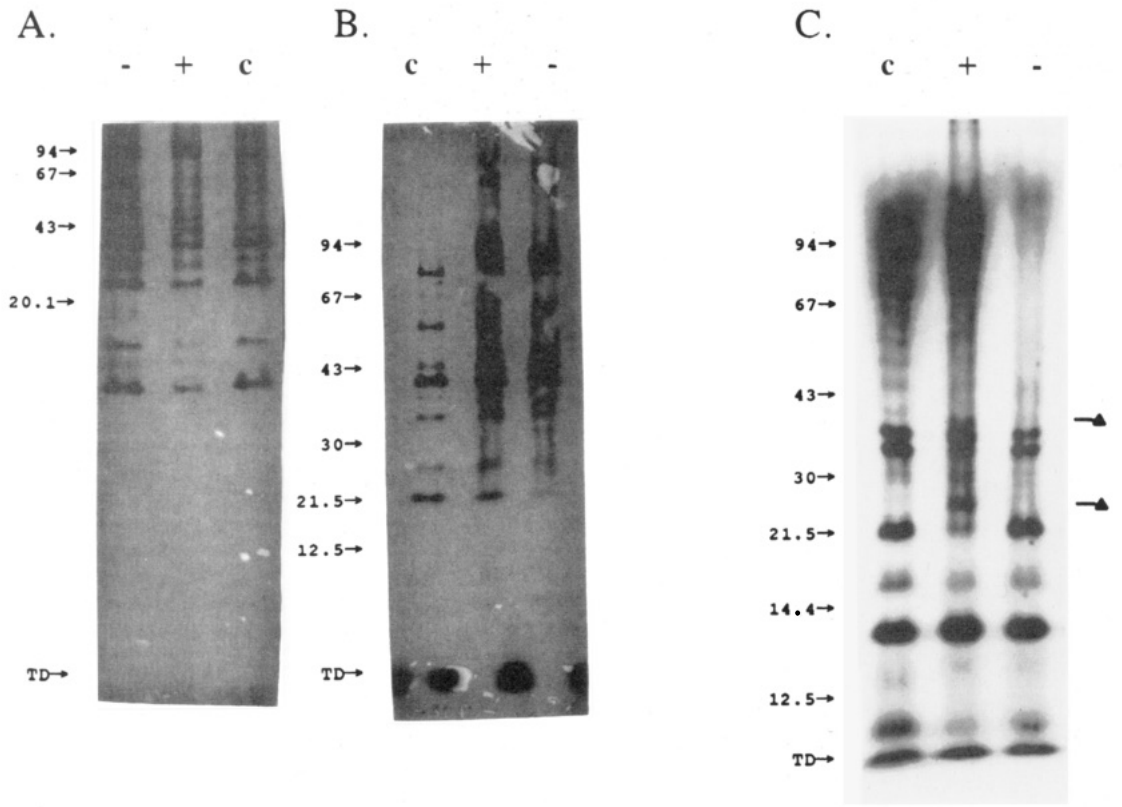

FIGURE 5: Random chemical cleavage of the cross-linked milli-calpain−TIPPEYRin complexes: MBS-modified TIPPEYRin was incubated with m-calpain in the presence of $CaCl_2$ (+) or with EGTA (−) for 30 min. An additional control (c) where m-calpain was incubated in the presence of calcium but with unmodified TIPPEYRin is also shown. Cross-linking was terminated, and cleavage at Cys residues using ^{14}CN was as described under Experimental Procedures. Panels A and B: Immunoblot detection of fragments containing the amino-terminal epitope. Cys fragments (16 μg) from each reaction were separated on 16.5% (panel A) or 8% (panel B) acrylamide gels, blotted, and probed with N-terminal-specific serum as described under Experimental Procedures. Panel C: Fluorographic detection of ^{14}C-labeled non-amino-terminal fragments. Samples were electrophoresed on an 8% gel, visualized by staining, and processed for fluorography. The film shown was exposed for 4 days with an intensifying screen. Two cleavage products (▲, 24.5 and 37 kDa) were unique to the calpain cross-linked in the presence of calcium (+). Molecular mass standards are labeled in kDa; TD indicates the tracking dye.

are three, highly conserved clusters, referred to as the central consensus sequence, and the N-terminal and C-terminal flanking sequences. Several prior studies have demonstrated the inhibitory activity of peptides based on the central consensus sequence (shown in Figure 1B) and shown this sequence to be both necessary and sufficient for specific inhibition of calpain. Structural studies demonstrated 11.8% α-helix and no β-sheet within calpastatin (Maki et al., 1988), and the core inhibitory peptide was predicted to be surface-exposed in intact calpastatin (Maki et al., 1988). Thus, despite calpastatin's large size (approximately 700 residues), its activity is heat-stable and small fragments of calpastatin, as well as synthetic peptides based on its conserved sequence, retain function (DeMartino et al., 1988; Emori et al., 1988; Maki et al., 1988, 1989; Kawasaki et al., 1989). These facts suggest that the tertiary structure of calpastatin is not a major factor in its interaction with calpain and it is reasonable to expect that peptide-mimics of calpastatin will bind to holo-calpain in a functional protein−protein interaction at, or near, the normal site of calpastatin-mediated inhibition. The relevancy of the TIPPEYRin cross-linking site to the authentic calpastatin binding site is supported by several key results. (1) Calpains are specifically inhibited by the peptide. (2) The MBS-modified peptide retains inhibitory activity. (3) Cross-linking is Ca^{2+}-dependent. (4) Calpastatin blocks the calcium-dependent cross-linking between TIPPEYRin and calpain. Although the intact calpastatin may have additional interactions with calpain, the ability of its most highly conserved sequence to inhibit enzyme activity, a function mimicked by TIPPEYRin, suggests that the peptides interaction with calpain is central to calpastatin function.

In the absence of sufficient enzyme protein to allow complete tryptic digestion for direct isolation and sequencing of the cross-linked products, a novel approach was required. Although limited proteolysis of the cross-linked, native calpain could produce a fragmentation pattern simpler than random chemical cleavage, the susceptibility of calpain to other proteases is altered by its calcium-dependent conformational changes (Croall, unpublished observation) that complicate analysis of those results. Although random chemical cleavage alone provides limited information, the N-terminal specific antiserum and incorporation of ^{14}C into the non-amino-terminal fragments allow mapping of the "landmark" cross-linking site within calpain. The N-terminal-specific antibody demonstrated that the calcium-specific site of cross-linking is at least 300−320 residues away from the amino terminus, i.e., not in domain I or most of domain II. Thus, cross-linking is not directly to the active-site Cys (C-104) or one proximal to it (C-97). This result is consistent with observations that E64 can still modify the active-site thiol in the presence of calpastatin (Kawasaki et al., 1990) and with the results we described for μ-calpain. Incorporation of ^{14}C into non-amino-terminal fragments demonstrated that the TIPPEYRin binding site is not within 22 kDa of the C-terminus, corresponding to approximately residues 510−701, domain IV of calpain. If the low molecular weight fragments are not C-terminal fragments (i.e., are produced by cleavage at more than one cysteine),

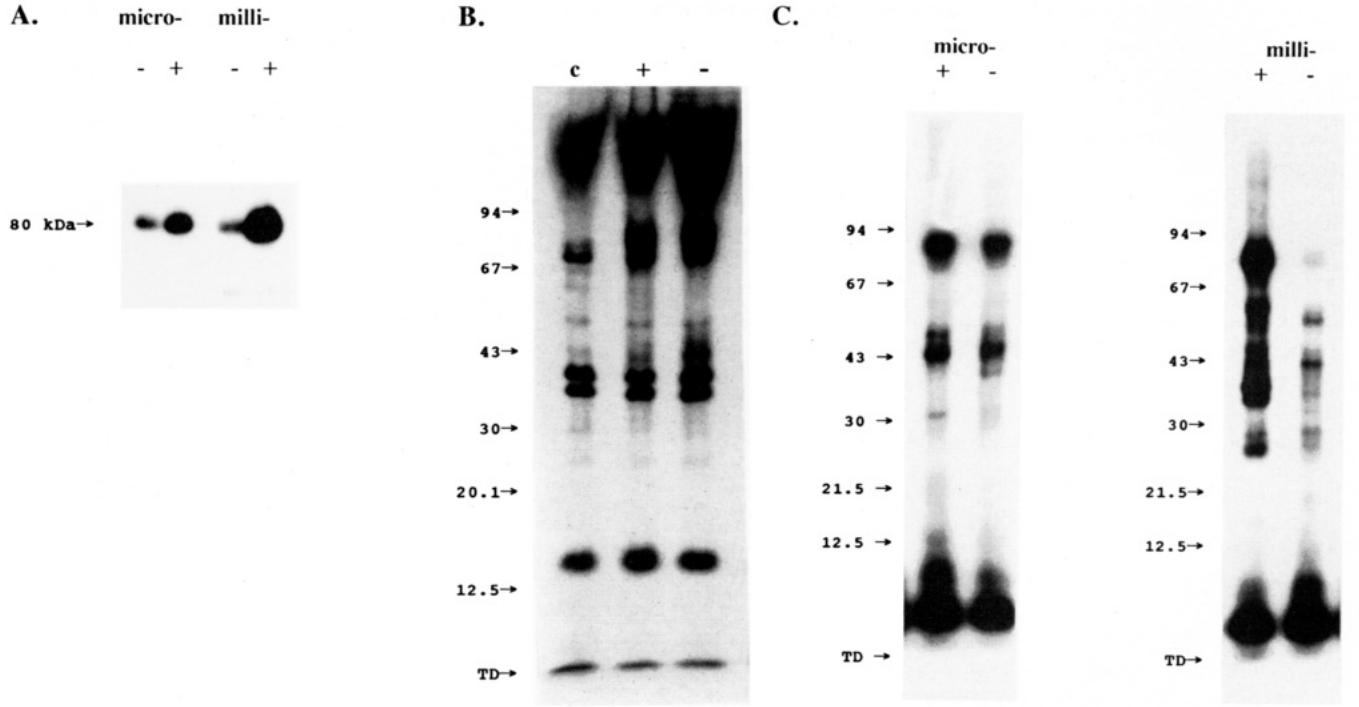

FIGURE 6: Cross-linking TIPPEYRin to micro-calpain. Panel A: ^{125}I-TIPPEYRin modified with MBS was cross-linked to micro- or milli-calpain for 30 min in the absence (−) or presence (+) of calcium and treated with SDS. The undigested cross-linked products migrating at approximately 80 kDa were visualized by autoradiography. Panel B: Micro-calpain cross-linked to TIPPEYRin in the presence (+) or absence (−) of calcium and from a mock (c) cross-linking reaction was analyzed by random chemical cleavage at cysteine residues. The non-amino-terminal fragments were labeled with ^{14}C, separated on an 8% acrylamide gel, and visualized by fluorography. Panel C: The cross-linked products from panel A were also processed to allow random chemical cleavage at cysteines. The resulting peptides were separated on an 8% acrylamide gel and ^{125}I-containing fragments visualized by autoradiography. Molecular mass standards migrated as shown (kDa).

this would imply that the site of cross-linking was even further from the C-terminal Ca^{2+} binding domain (domain IV). These results allow us to localize the functional interaction between TIPPEYRin and calpain within domain III, approximately residues 300 through 510.

In the presence of calcium, cross-linking of TIPPEYRin to bovine μ-calpain also occurred. However, in contrast to results with m-calpain, no specific site of cross-linking could be demonstrated within μ-calpain. This suggests that although the peptide−protein interaction is favored by the presence of calcium, no single cysteine is appropriately aligned with the peptide-linked maleimide for rapid reaction. Therefore, the peptide becomes cross-linked randomly to several less favorable cysteines. This result is important for two reasons. First, it demonstrates that the methods we have used can distinguish between cross-linking to a specific site and random distribution of the peptide-linked maleimide with reactive thiols. Second, it indicates that the specific cross-link to milli-calpain must not be at any of the nine completely conserved Cys but requires a cysteine specific to m-calpain. Only Cys-497 in domain III [a Cys present in rat (DeLuca et al., 1993), pig (Sun et al., 1993), rabbit, and human m-calpains but not in μ-calpain or chicken calpain (Mellgren & Murachi, 1990)] is consistent with all of our results. The sequences surrounding this Cys are highy conserved as would be expected for the calpastatin binding site (20/29 residues are identical, and 7 additional positions maintain conservative substitutions between chicken, rabbit, and human). We are in the process of raising C-terminal peptide-specific antisera to confirm the site of cross-linking.

In a prior study (Nishimura & Goll, 1991), autoproteolytic fragments of calpain were passed through a calpastatin affinity column, and the calcium binding domains (IV and IV′) were bound in a calcium-dependent interaction. However, Ca^{2+}-dependent binding of enzyme fragments to the calpastatin affinity column may not have any direct relationship to functional inhibition because the structures of calpain fragments may differ significantly from their functional conformations. Binding could result from changes in hydrophobicity of the isolated Ca^{2+} binding domains when calcium is bound, or apparent binding may result from solubility changes of these peptides (Crawford et al., 1990). A recent report, in fact, demonstrated the lack of a correlation between the Ca^{2+}-dependent binding of various calpastatin domains to the calmodulin-like (domain IV) regions of calpain and the ability of the calpastatin domain to function as an inhibitor (Ma et al., 1993). This suggests that the previously observed binding may not be related to the functional interaction between the two proteins. The experimental approach reported here to identify the binding site for calpastatin is therefore preferable to that previously described, despite its own inherent drawbacks. For example, cross-linking with MB-TIPPEYRin is biased to the extent that it requires a reactive cysteine and the calcium binding domain of the smaller, regulatory subunit is Cys-poor relative to the other domains. Our results cannot exclude the possibility that calpastatin may also interact with the calmodulin-like, calcium binding domains of either or both subunits as was previously suggested (Nishimura & Goll, 1991). However, our results demonstrate that the binding site for a peptide that mimics calpastatin is sufficiently proximal to domain III of the catalytic subunit to be specifically cross-linked to it. Although the exact sequence of the catalytic subunit of the bovine enzyme is not known, conservation of functionally important regions is well documented. The highly conserved consensus sequence and

function of calpastatin suggest a conserved binding site, and thus bovine enzyme can provide useful information for these structure−function studies. From studies using 34-mers identical to each of the four repeated central consensus sequences of calpastatin, Kawasaki et al. suggested that the tighter binding (IC_{50} = 0.120−0.016 μM) of the first and fourth calpastatin functional regions may be attributed to two adjacent leucine residues (Kawasaki et al., 1989). These leucines correspond to residues 17 and 18 within the TIPPEYRin peptide described in this report. Peptides capable of inhibiting calpain appear to require at least LGXXXXTIPPXYRXLL for an IC_{50} less than or equal to a micromolar range (Kawasaki et al., 1989). Because the calpastatin-related peptides act as competitive inhibitors, further description and characterization of the calpastatin binding site may also provide insight into calpain's requirements for substrate recognition and selection at sites distant from the catalytic residues.

ACKNOWLEDGMENT

We thank Deborah Reid, Scott Despres, and Colleen Skelly for technical assistance in various phases of this work and Brian Higgins, NPS Studios, for production of Figures 3−6. Special thanks to M. Spencer and J. Tidball (UCLA) for the antiserum against conserved calpain residues 282−301 (chicken sequence); R. Gundersen, University of Maine, and G. N. DeMartino, Southwestern Medical School, for critical reading of the manuscript; and the anonymous reviewers for *Biochemistry* for their important and useful comments.

REFERENCES

Adachi, Y., Maki, M., Ishii, K., Hatanaka, M., & Murachi, T. (1989) *Adv. Second Messenger Phosphoprotein Res. 24*, 478−484.

Aoki, K., Imajoh, S., Ohno, S., Emori, Y., Koike, M., Kosaki, G., & Suzuki, K. (1986) *FEBS Lett. 205*, 313−317.

Beckerle, M. C., Burridge, K., DeMartino, G. N., & Croall, D. E. (1987) *Cell 51*, 569−577.

Bradford, M. (1976) *Anal. Biochem. 72*, 248−253.

Brumley, L. M., & Wallace, R. W. (1989) *Biochem. Biophys. Res. Commun. 159*, 1297−1303.

Choi, K. Y., Kim, H. A., Lee, S. Y., Moon, K. Y., Sim, S. S., Kim, J. W., Chung, H. K., & Rhee, S. G. (1990) *Science 248*, 64−66.

Cong, J., Goll, D. E., Peterson, A. M., & Kapprell, H. P. (1989) *J. Biol. Chem. 264*, 10096−10103.

Coolican, S. A., Haiech, J., & Hathaway, D. R. (1986) *J. Biol. Chem. 261*, 4170−4176.

Cottin, P., Poussard, S., Desmazes, J. P., Georgescauld, D., & Ducastaing, A. (1991) *Biochim. Biophys. Acta 1079*, 139−145.

Crawford, C., Brown, N. R., & Willis, A. C. (1990) *Biochem. J. 265*, 575−579.

Croall, D. E. (1989) *Biochemistry 28*, 6882−6888.

Croall, D. E., & DeMartino, G. N. (1984) *Biochim. Biophys. Acta 788*, 348−355.

Croall, D. E., & DeMartino, G. N. (1990) in *Intracellular Calcium-Dependent Proteolysis* (Mellgren, R. L., & Murachi, T., Eds.) pp 103−114, CRC Press, Boca Raton FL.

Croall, D. E., & DeMartino, G. N. (1991) *Physiol. Rev. 71*, 813−847.

Croall, D. E., Slaughter, C. A., Wortham, H. S., Skelly, C. M., DeOgny, L., & Moomaw, C. (1992) *Biochim. Biophys. Acta 1121*, 47−53.

DeLuca, C. I., Davies, P. L., Samis, J. A., & Elce, J. S. (1993) *Biochim. Biophys. Acta 1216*, 81−93.

DeMartino, G. N., & Croall, D. E. (1984) *Arch. Biochem. Biophys. 232*, 713−720.

DeMartino, G. N., Huff, C. A., & Croall, D. E. (1986) *J. Biol. Chem. 261*, 12047−12052.

DeMartino, G. N., Wachendorfer, R., McGuire, M. J., & Croall, D. E. (1988) *Arch. Biochem. Biophys. 262*, 189−198.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70−77.

Emori, Y., Kawasaki, H., Imajoh, S., Minami, Y., & Suzuki, K. (1988) *J. Biol. Chem. 263*, 2364−2370.

Fox, J. E. B., Austen, C. D., Reynolds, C. C., & Steffen, P. K. (1991) *J. Biol. Chem. 266*, 13289−13295.

Frangioni, J. V., Oda, A., Smith, M., Salzman, E., & Neel, B. G. (1993) *EMBO J. 12*, 4843−4856.

Giancotti, F. G., Strepp, M. A., Suzuki, S., Engvall, E., & Ruoslahti, E. (1992) *J. Cell Biol. 118*, 951−959.

Graberek, Z., & Gergely, J. (1990) *Anal. Biochem. 185*, 131−135.

Hall, T. G., & Bennett, V. (1987) *J. Biol. Chem. 262*, 10537−10545.

Harris, A. S., Croall, D. E., & Morrow, J. S. (1988) *J. Biol. Chem. 263*, 15754−15761.

Harris, A. S., Croall, D. E., & Morrow, J. S. (1989) *J. Biol. Chem. 264*, 17401−17408.

Imajoh, S., Aoki, K., Ohno, S., Emori, Y., Kawasaki, H., Sugihara, H., & Suzuki, K. (1988) *Biochemistry 27*, 8122−8128.

Inomata, M., Kasai, Y., Nakamura, M., & Kawashima, S. (1988) *J. Biol. Chem. 263*, 19783−19787.

James, P., Maeda, M., Fischer, R., Verma, A. N., Krebs, J., Penniston, J. T., & Carafoli, E. (1988) *J. Biol. Chem. 263*, 2905−2910.

Kawasaki, H., Emori, Y., Imajoh-Ohmi, S., Minami, Y., & Suzuki, K. (1989) *J. Biochem. 90*, 1787−1793.

Kawasaki, H., Emori, Y., & Suzuki, K. (1990) in *Protease Inhibitors* (Takada, A., et al., Eds.) pp 189−199, Elsevier Science Publishing Co., New York.

Kishimoto, A., Mikawa, K., Hashimoto, K., Yasuda, I., Tanaka, S., Tominaga, M., Kuroda, T., & Nishizuka, Y. (1989) *J. Biol. Chem. 264*, 4088−4092.

Laemmli, U. K. (1970) *Nature 227*, 680−685.

Litersky, J. M., & Johnson, G. V. W. (1992) *J. Biol. Chem. 267*, 1563−1568.

Ma, H., Yang, H. Q., Takano, E., Lee, W. J., Hatanaka, M., & Maki, M. (1993) *J. Biochem. 113*, 591−599.

Maki, M., Takano, E., Osawa, T., Ooi, T., Murachi, T., & Hatanaka, M. (1988) *J. Biol. Chem. 263*, 10254−10261.

Maki, M., Bagci, H., Hamaguchi, K., Ueda, M., Murachi, T., & Hatanaka, M.(1989) *J. Biol. Chem. 264*, 18866−18869.

McClelland, P., Lash, J. A., & Hathaway, D. R .(1989) *J. Biol. Chem. 264*, 17428−17431.

Mellgren, R. L., & Murachi, T. (1990) *Intracellular Calcium-Dependent Proteolysis*, pp 3−274, CRC Press, Boca Raton, FL.

Nefsky, B., & Bretscher, A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3549−3553.

Neuhoff, V., Arnold, N., Taube, D., & Ehrhardt, W. (1988) *Electrophoresis* 9, 255−262.

Nishimura, T., & Goll, D. E. (1991) *J. Biol. Chem. 266*, 11842−11850.

Schagger, H., & vonJagow, G. (1987) *Anal. Biochem. 166*, 368−379.

Sun, W., Ji, S. Q., Ebert, P. J., Bidwell, C. A., & Hancock, D. L. (1993) *Biochimie 75*, 931−936.

Sutoh, K. (1982) *Biochemistry 21*, 3654−3661.

Suzuki, K., & Ohno, S. (1990) *Cell Struct. Funct. 15*, 1−6.

Suzuki, K., Imajoh, S., Emori, Y., Kawasaki, H., Minami, Y., & Ohno, S. (1987) *FEBS Lett. 220*, 271−27745.

Wang, K. K. W., Villalobo, A., & Roufogalis, B. D. (1989) *Biochem. J. 262*, 693−706.