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Detecting the Active Conformation of Calpain with Calpastatin-Based Reagents

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SUMMARY

The specific, calcium dependent, high affinity interaction between calpain and its endogenous inhibitor calpastatin was exploited to selectively detect the calcium-bound, catalytically competent, conformation of calpain in vitro. Modification of calpastatin-domain-1 (Val₁₁₄-Ser₂₇₀) or its N-terminal fragment (Val₁₁₄-Pro₂₀₂), at selected unique cysteine residues with maleimide-AlexaFluor546 did not compromise calpastatin function (inhibition of calpain) or its binding with calpain. Ca²⁺-dependent binding between catalytically-dead calpain-2 (Cys₁₀₅Ala) fused with eGFP and these fluorogenic calpastatin peptides generates fluorescent resonance energy transfer (FRET). The FRET signal documents proximity of calpain-2-C-terminally linked fluorophore to specific sites within calpastatin when the proteins form a complex. These results provide important insights into the calcium dependent interaction between calpain and calpastatin and for holo-calpain-2 in solution experimentally validate some key features of their predicted interactions. These data also provide proof of concept that the calpastatin based reagents may be useful to selectively detect the active conformation of calpain.

Keywords

calpastatin; calpain; FRET; protease

1. Introduction

The physiological significance of the classical, ubiquitous, calcium-dependent, cysteine proteinase calpain-2 (EC 3.4.22.53) is unequivocally established through results of targeted gene deletion experiments. Disruption of genes encoding calpain's small subunit (cpns-1/capn4) [1,2] or the catalytic subunit of calpain-2 (capn2) [3] is lethal early in murine embryogenesis implying that calpain-2 performs some essential, but as yet undefined function in early development. The ubiquitous classical calpains -1 and -2 (also known as μ - and m - respectively) [4,5] also participate in a variety of signal transduction pathways leading to cell motility, cell transformation, invasion and cell death as evidenced by studies using numerous cellular, tissue or organismal models [4–7]. Much has been learned about the biochemistry of

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these enzymes and structural details are now available for the penta- EF hand module (dVI) within the small subunit [8,9] and the enzymes' catalytic core (dI and dII) [10] in both the presence and absence of calcium. These data in conjunction with structures of heterodimeric calpain-2 and calpain-1-like enzymes in the absence of calcium [11–13] (their inactive conformation) greatly contribute to the understanding of calpain; knowledge that will be important for designing approaches to reveal the enzyme's specific biological roles.

Distinguishing the small fraction of active enzyme from the background population of calpain will be important for determining its physiological functions. A few activity based methods have been suggested to demonstrate calpain function in situ [14–16]. However, typically the key evidence that the observed proteolysis is catalyzed by calpain requires measuring differences in signal obtained in the presence versus the absence of imperfect protease inhibitors. Selective depletion of a calpain through antisense RNA or siRNA contributes significantly to elucidation of specific roles for individual calpain isoforms in signal transduction pathways [17,18] and may identify potential downstream targets. These studies, however, do not directly identify activating signals for the enzymes. Small molecule inhibitors are often key for dissecting the functions of proteolytic enzymes and were successfully applied to reveal details about caspases [19]. Unfortunately, calpains strongly prefer protein substrates relative to peptides and cleavage sites represent an array of sequences suggesting little constraint from primary sequence at susceptible bonds. Thus desirable, high affinity, high specificity, small molecule inhibitors remain elusive and new reagents are needed to investigate the cellular mechanisms that activate and regulate calpains.

Calpastatin, the specific, proteinaceous, endogenous inhibitor of calpain, is an attractive protein to exploit as a potential selective reagent for several reasons. First, calpastatin selectively binds to the calcium bound, active conformation of the two best characterized calpains: 1 (or μ) and 2 (or m) with high affinity [5,20]. Second, it has four homologous inhibitory domains each characterized by highly conserved peptide sequences designated A, B and C (Figure 1A). Significantly each of the conserved calpastatin peptides binds, independently, in a calcium dependent interaction with calpain (peptides A, B and C bind) or with the penta-EF hand domains dIV and/or dVI (peptides A and C bind) with reasonably high affinity (~ 10 – 80 nM [20–25]). Third, calpastatin is a cysteine poor, intrinsically unstructured (disordered) protein making it amenable to the introduction of uniquely positioned cysteine residues. Chemical modification of judiciously selected residues with maleimide fluorophores is predicted not to disrupt calpastatin binding or function. Perhaps most importantly, the structure of a calpain-dVI- in the presence of calcium and complexed with a synthetic 19-mer peptide mimic of the calpastatin C peptide [9] (Figure 2A) provides a model for binding of calpastatin's A and C conserved peptides to the homologous domains IV and VI of calpain (Figure 2A and B). Based on this structure we hypothesized that a C-terminal fluorophore on calpain could demonstrate calcium-dependent, fluorescent resonance energy transfer (FRET) with an appropriate partner fluorophore positioned within an engineered fragment of calpastatin when the enzyme and inhibitor bind because the structure predicts proximity (~ 26 Å) of the C-terminus of calpain to specific regions of the calpastatin peptide. A schematic of the component proteins and their expected binding interactions tested for FRET is shown in Figure 2C. If binding produces FRET this will specifically indicate the presence of the catalytically competent conformation of calpain. Data reported here provide direct evidence to demonstrate 1) that fluorogenic modification of calpastatin peptides at selected residues does not interfere with their calcium dependent binding interactions with calpain; 2) that the predicted interactions between calpain and a functional domain of calpastatin do occur in solution with holo-calpain-2; and 3) proof of concept that fluorogenic calpastatin peptides may be exploited to selectively detect the active conformation of calpain. modified to contain a C terminal fluorophore such as eGFP.

2. Materials and Methods

Human calpastatin pIND(SP1)/V5-His C was generously provided by Dr. Masatoshi Maki (Nagoya University, Japan) and Dr. Ronald Mellgren (Medical College of Ohio). Plasmids containing cDNA encoding calpain-2 -Cys₁₀₅Ala (rat) in pET24 (Novagen) and the truncated small subunit (A₉₄-S₂₇₀ – rat cpns1/capn4 gene product) in a plasmid designated pACpET were generously provided by Dr. John Elce (Queens University, Ontario, Canada). Plasmid peGFP was obtained from Clontech. Tetra-methylrhodamine-5-maleimide, AlexaFluor 546-5-maleimide and LumioGreen were purchased from Molecular Probes/Invitrogen. Bradford reagent and porcine erythrocyte calpain 1 were from Calbiochem. Bicinchoninic acid (BCA) assay reagents were from Pierce. Ethane-dithiol (EDT) and most other reagents were from Sigma-Aldrich.

2.1 Subcloning and site directed mutagenesis of calpastatin

To subclone the cDNA encoding human calpastatin, it was amplified by polymerase chain reaction (PCR) from the template pIND(SP1)/V5-HisC using primers to vector sequence (5'-GCTCGGATCCA-CTAGTCCAG-3'; 5'-GCCACTGTGCTGGATATCTG-3') that flanked the insertion site, cleaved with EcoR1 and ligated into a Bluescript plasmid (Stratagene). The resulting plasmid provided template for subcloning a single domain of calpastatin (Val₁₁₄ to Ser₂₇₀) into peT24 (Novagen). The desired domain was amplified by PCR using primers extended with a 5' NdeI site (that also provides an initiator Met) and 3' EcoRI site (5'-CGATACCGGC-TGCATATGGTTGCTGGTATCACTGCAATATC-3'; 5'-GCCTGAGAATTC-ACTTCTGACTGTCCCTGCT-3'). This construct encodes 34 residues of the carboxy-end of calpastatin's L domain (cL), the highly conserved A-B-C peptides of the functional domain 1 and vector residues including a hexa-histidine (H6) tag. The resulting peptide is designated cL-ABC-D1 (Figure 1B). Variants of cL-ABC-D1 were produced via QuikChange II XL site directed mutagenesis (Stratagene) to contain no cysteine (Cys₂₄₁Ser; TGT→AGT) or a single unique cysteine residue as Ser₁₄₂Cys, TCT→TGT; Ser₁₄₈Cys, TCG→TGC; Ser₁₅₁Cys, TCA→TGT; Ser₂₂₃Cys, TCA→TGT; and Ala₂₄₆Cys, GCT→TGT. The thiol free expressed peptide provided a control for any nonspecific reaction with the maleimide fluorophores. QuikChange mutagenesis was done with plasmid encoding thiol free cL-ABC-D1 as template (primers 5'-GAAGTCACAATTCCT(^)GAAT-TCGAGCTCCACAAGC-3' and its complement) to delete 204 nucleotides (encoding 68 amino acid residues; Pro₂₀₃ to Ser₂₇₀) such that the expressed calpastatin product includes the cL residues, a truncated B peptide and deletes all of the C peptide while maintaining the vector encoded H6 tag. These calpastatin fragments are designated cL-AtB-D1 and include variants with Ser₁₄₂Cys, or Ser₁₄₈Cys, or Ser₁₅₁Cys (Figure 1C). To create a plasmid to express a calpastatin fragment containing only the conserved C-peptide of domain-1, mutagenesis was performed to generate a new NdeI site (TAT→ATA), to encode an initiator Met immediately N-terminal to Ala₂₁₀. Removal of the NdeI-NdeI fragment from the plasmid deleted Val₁₁₄-Leu₂₀₉ from the encoded protein. A mutagenesis reaction using template encoding thiol free peptide changed Ala₂₄₆Cys, GCT→TGT using 5'-CACCAGTGGGTCGCTACATGTTGCTGGAAAGAAACTG-3' as the sense primer of the mutagenic primer pair. The resulting peptides are designated as C-D1 with Cys₂₄₁ (natural sequence), Cys₂₄₁Ser, (thiol-free control); Cys₂₄₁Ser- Ser₂₂₃, or 246 or 256 Cys (Figure 1D).

2.2 Subcloning and mutagenesis of the catalytic subunit of calpain-2

Deletion mutagenesis (using the primer 5'-GCTGAGTTTTTCAGTACTCCTG(^)AAGCTTGCGGCC-3 and its complement) linked the vector encoded his-tag to the catalytically inactive calpain 2-Cys₁₀₅Ala (rat). In preparation for labeling with LumioGreen, DNA encoding AlaCysCys-ProGlyCysCysAla (designated CCPGCC) was inserted at the 3' end of C₁₀₅A-calpain-2 cDNA with the primer 5'-GCCGCACTCGAG-

GCCTGCTGCCCCGGGTGCTGCGCCCA-CCACCACCAC-3' and its complement. To fuse eGFP to the C-terminus of calpain, the pEGFP vector was digested with HindIII and NotI and ligated into those sites between calpain-2 cDNA and the hexa-His tag. A two base insertion (5'-CGAGCTGTACAAG**GGT**AAA-GCGGCCGCAC-3' and its complement) allowed linkage to the his-tag C-terminal to calpain-eGFP.

2.3 Expression and purification of calpain and calpastatin

Escherichia coli strain BL21(DE3) (Novagen) was co-transformed with each plasmid variant encoding calpain's catalytic subunit and pACpET encoding the truncated calpain small subunit (rat) [8,26] with kanamycin (10 µg/mL) and ampicillin (50 µg/mL) selection. Similarly *Escherichia coli* strain BL21(DE3) were transformed with plasmids encoding calpastatin fragments using kanamycin (10 µg/mL) selection. Isopropyl-beta-D-thiogalactopyranoside (IPTG) (0.5mM) induced expression of recombinant proteins for 14–18 hrs at room temperature. Cells were harvested, washed and stored at –80° C prior to lysis. For enzyme purification frozen cell pellets were resuspended in 50 mM Mops pH 7.5, 10 mM EGTA, 10 mM EDTA, 10 mM β-mercaptoethanol (β-ME) (buffer A) (~4–8: 1 vol: wet weight), sonicated (Branson Sonifier 450) on ice in the presence of phenylmethylsulfonyl fluoride (PMSF) (50 µg/mL) and centrifuged (35k × g at 4°C for 20 minutes) to generate the soluble fraction containing calpain. Total protein was quantified by Bradford assay [27] and concentration of calpain Cys₁₀₅Ala-eGFP was also determined at A508nm using $\epsilon = 55,900 \text{ cm}^{-1} \text{ M}^{-1}$ for eGFP. To purify calpain the resulting soluble proteins were fractionated on DEAE Sephacel. The unbound protein and proteins eluted at 0.15M NaCl in modified buffer A (chelators reduced to 2mM each) were discarded prior to elution of calpain variants with 50 mM Mops pH 7.0, 0.5 M NaCl, 2 mM EGTA, 2 mM EDTA, 5 mM β-ME (Buffer B). The eluted protein was immediately chromatographed on Reactive Red Agarose (RRA) as described previously [28]. For calpains containing the H6 affinity tag, samples were further purified on Ni NTA agarose (Qiagen). Binding conditions included 10mM imidazole in 25 mM Mops pH 8.0, 0.10 M NaCl, 0.4 mM EGTA, 0.2 mM EDTA, 0.1 mM β-ME (buffer C) and calpain was eluted by increasing imidazole to 250 mM in buffer C. Purified calpains and partially purified calpain-Cys₁₀₅Ala-eGFP were dialyzed against 50 mM Mops pH 7.5, 1 mM EGTA, 1 mM EDTA, 0.5 mM dithiothreitol (DTT). Purified calpain -Cys₁₀₅Ala-CCPGCC was dialyzed against 50 mM Mops pH 7.5, 1 mM Tris (2-carboxyethyl) phosphine (TCEP) prior to modification with LumioGreen (see below). Aliquots of enzyme were flash frozen using liquid nitrogen, and stored at –80°C until thawed for use.

For isolation of calpastatin peptides, the bacterial cell pellets were resuspended in Buffer C containing 10mM imidazole and subjected to three freeze (–80°C) –thaw cycles. The supernatant obtained by centrifugation (35k × g at 4°C for 20 min) was chromatographed on Ni-NTA agarose for purification of the his-tagged proteins that eluted with 100 mM imidazole in Buffer C. The eluted protein was dialyzed against 50 mM Hepes pH 7.0, 1 mM TCEP. Prior to fluorogenic modification, purified calpain and calpastatin were examined by SDS-PAGE with a Tris-Tricine buffer system [29]. Representative results are shown in Figure 3A. Proteins were visualized with 0.1% Coomassie blue, 0.05% amido black, in 40% methanol, 10% acetic acid and images of gels were recorded using the Chemimager 4400 Low Light Imaging System (Alpha Innotech Corporation). Images were saved in digital format (jpg or tiff files) and images provided accurately represent the original stained gels.

2.4 Fluorogenic modification of calpastatin-variants and calpain-Cys₁₀₅Ala -CCPGCC-H6

The calpastatin peptides were labeled using recommendations from Molecular Probes - Invitrogen. Tetramethyl-rhodamine-5-maleimide (TMR) or AlexaFluor 546-5-maleimide were solubilized in dimethyl-sulfoxide (DMSO). Fluorophore-maleimide was added dropwise to achieve a 5–7-fold molar excess relative to calpastatin-peptide (100–200 µM) and reactions

were incubated for 1 hour at ambient temperature. The addition of β -ME (5 fold molar excess to maleimide) terminated the reaction. AlexaFluor- or TMR-labeled peptides were dialyzed extensively against 25 mM Mops pH 7.5 using Tube-O-Dialyzers (Genotech). To estimate stoichiometry of protein modification, fluorophore concentration was measured by absorbance using $\epsilon = 95,000 \text{ cm}^{-1} \text{ M}^{-1}$ for TMR at 541nm and $\epsilon = 93,000 \text{ cm}^{-1} \text{ M}^{-1}$ for AlexaFluor546 at 554 nm and expressed relative to the molar concentration of peptide, prior to labeling, as estimated by A280 ($\epsilon = 4675 \text{ cm}^{-1} \text{ M}^{-1}$ for cL-ABC-D1 and $\epsilon = 3610 \text{ cm}^{-1} \text{ M}^{-1}$ for cL-AtB-D1); Bradford reagent (Biorad) and/or the enhanced bicinchoninic acid (BCA) assay (Pierce). Thiol-free variants of calpastatin fragments (cL-ABC-D1 and cL-AtB-D1) were also treated with maleimide fluorophores to provide experimental controls for potential non-specific labeling of the constructs. Control peptides were labeled in the absence of TCEP thus favoring more efficient label incorporation than modification reactions in the presence of TCEP [30, 31]. Fluorogenically modified peptides were stored at -80°C and can be subjected to freeze-thaw cycles without any apparent change to their function.

To specifically label calpain-Cys₁₀₅Ala containing the C-terminal CCPGCC-motif known to be a binding site for LumioGreen [32], a solution of 1 mM LumioGreen, 10 mM EDT, 92% DMSO was prepared immediately prior to mixing with enzyme and fluorophore added to 2 moles per mole of calpain in 50 mM Mops pH 7.5, 1 mM TCEP with the final concentration of DMSO $\leq 1\%$ and incubated 16–18 hrs at 4°C . LumioGreen labeled calpain was dialyzed extensively against 50 mM Mops, pH 7.5, 1 mM EGTA, 1 mM EDTA, 0.5 mM DTT and the stoichiometry of modification was determined to be $\sim 0.5:1$; $\epsilon = 41,000 \text{ cm}^{-1} \text{ M}^{-1}$ at 508 nm for LumioGreen. Aliquots of calpain-Cys₁₀₅Ala-LumioGreen were flash frozen and stored at -80°C and thawed only once.

2.5 Assessing FRET as an indicator of calpain-calpastatin binding

Binding assays (260–750 μL in Starna cuvettes) were conducted at ambient temperature ($22\text{--}28^{\circ}\text{C}$) at total protein concentrations of $\geq 0.2 \text{ mg/mL}$ in buffer pH 7.5 (either 50 mM Hepes or Mops), 0.15–0.25 M NaCl and reducing agent, 1 mM TCEP or 0.5 mM DTT. Bovine serum albumin (BSA) was added to some assays to maintain total protein concentration. A Perkin-Elmer LS50 B luminescence spectrometer was used for excitation of samples at 460nm and collection of emission scans between 480nm to 640nm. Slit widths were varied between experiments (4.0–7.0) but remained constant within a given experiment. Figure 4 outlines the basic assay design for data collection of emission scans: (i) the donor, i.e. calpain-Cys₁₀₅Ala-eGFP- (0.3–1 μM) or calpain-Cys₁₀₅Ala-LumioGreen (2 μM) in the presence of added non-fluorescent proteins; (ii) after addition of acceptor labeled calpastatin peptides (0.5–5 μM); (iii) after addition of calcium (from 0.005–10 mM), and after reactions were terminated by the (iv) addition of EGTA (to 18–20 mM) to show reversibility of the binding and loss of the FRET signal. For each assay condition, five replicate scans were collected and data were imported into MatLab5.3 (Mathworks.com) for analysis. The average of each set of 5 scans was used for determining emission ratios. The ratio of the intensity of the acceptor emission peak to the intensity of the donor emission peak ($\text{Em}_{\text{Acc}} \sim 570\text{nm} / \text{Em}_{\text{Don}} \sim 508\text{nm}$) was calculated as a measure of FRET. The increase in $\text{Em}_{\text{Acc}} / \text{Em}_{\text{Don}}$ in response to calcium was calculated as % increase = $(\text{Em ratio in the presence of calcium}) - (\text{Em ratio in the absence of calcium}) / (\text{Em ratio in the absence of calcium})$. Similarly the loss of FRET in response to EGTA was calculated as $(\text{Em ratio in the presence of calcium}) - (\text{Em ratio in the presence of EGTA}) / (\text{Em ratio in the presence of calcium})$. Control assays were performed with nonspecifically labeled (thiol free) acceptor-peptides to ensure that any nonspecifically labeled material was not responsible for the changes measured.

Concentrations of calcium stocks were validated by the Quantichrom™ calcium assay (Bioassaysys, Hayward, CA). The concentration of free calcium was calculated via

WEBMAXC Standard (Maxchelator) for experiments examining calcium as a variable. Calcium dependent changes in Em_{Acc}/Em_{Don} were calculated by subtracting Em_{Acc}/Em_{Don} measured in the absence of calcium from Em_{Acc}/Em_{Don} at a given calcium concentration. The average maximal calcium dependent change in Em_{Acc}/Em_{Don} was taken as 100% to allow comparison across several experiments. For determining FRET efficiency, donor fluorescence in the presence of calcium and acceptor was corrected for dilution and bleaching for comparison with initial intensity of the isolated donor. Data are given as means $\pm$ the standard deviation. For competition assays, the data were collected in non-sequential order to minimize any variation resulting from the order of data collection.

RESULTS

3.1 Ca^{2+} -dependent FRET between calpain-eGFP and a functional domain of calpastatin

Modification of calpain to serve as a potential FRET donor—Two strategies were used to add C-terminal fluorophores to calpain to provide the donor fluorophore for FRET; calpain-Cys₁₀₅Ala -fused with eGFP-H6 and calpain-Cys₁₀₅Ala-CCPGCC-H6 labelled with LumioGreen. It is well established that C-terminal modifications of catalytically competent calpain-2 do not impair enzyme activity [33] and casein zymography confirmed this by demonstrating activity for calpain Cys₁₀₅-CCPGCC-H6 (data not shown). Use of the catalytically dead calpain-Cys₁₀₅Ala in binding studies eliminated any potential for autoproteolysis and the need for inclusion of protease inhibitors.

Modification of calpastatin domain-1 to serve as potential acceptors for FRET

—The C-terminal 34 residues of the L domain (cL) and the highly conserved ABC peptides of inhibitory domain 1 of calpastatin (human, residues 114–270) (cL-ABC-D1, Figure 1B) and its N-terminal fragment (cL-AtB-D1, residues 114–202, Figure 1C), were used for initial binding studies. Variants of these calpastatin fragments were generated to lack cysteine (Cys₂₄₁Ser), or to contain a single cysteine residue at each of several positions as indicated (Figure 1) to allow for selective modification of the single thiol with maleimide-dyes. Estimates of labelling stoichiometry were dependent upon the method used to quantify calpastatin because calpastatin protein is typically underestimated by many protein assays [34, 35]. When calpastatin concentration was estimated by dye -binding based assays (e.g. Bradford or BCA) fluorophore incorporation was estimated as ~3–4:1. Using protein concentration determined by A280, the expected ~1:1 incorporation of fluorophore was measured for cL-AtB-D1-variants containing unique cys residues, but stoichiometry for cL-ABC-D1 was ~2:1. Because non-specific labeling of the α amino group of short peptides and/or lysine side chains [30, 36] is possible, peptides lacking cysteine (cL-ABC-D1-Cys₂₄₁Ser and cLAtB-D1) were also treated with maleimide-fluorophores and surprisingly also yielded high estimates of label incorporation (~2:1). Analysis of cys-containing and thiol-free labeled peptides by SDS-PAGE revealed strong fluorescence associated with cys containing peptides and no detectable fluorophore bound to the thiol free peptides, confirming that fluorophore was linked only to unique cysteines. (Figure 3B). There were however dye- containing lower molecular weight components revealing that the stoichiometry estimates were artifacts. To exclude the possibility that FRET related to the low molecular weight, but non-dialyzable fluorophore, binding assays were also conducted with these fluorogenic control peptides.

Although chemical modification has inherent potential to disrupt protein-protein interactions, the calpastatin residues selected for modification are proximal to, but not within, the core A and C induced helical regions that likely mediate the most critical interactions between calpain and calpastatin. The judicious choice of target residues increased the likelihood that the fluorogenic dye would not adversely affect binding or function. Traditional biochemical assays demonstrated that cL-ABC-D1 and its AlexaFluor-546 derivatives (with C₂₄₁S, S₂₂₃C or

S₂₄₆C) inhibited calpain-catalyzed hydrolysis of casein with an IC₅₀ = 20–50nM. Additionally cL-AtB-D1 and a fluorogenic derivative (C₁₄₈-AlexaFluor546) also inhibited calpain activity. As cL-AtB-D1 lacks part of the conserved essential B peptide and all of the C peptide it is not surprising, that it is a less potent inhibitor (IC₅₀ = 200–250 nM) than the complete domain, cL-ABC-D1. Based on these traditional assays of calpastatin function, the fluorogenic modification of the residues selected was not detrimental.

Binding assays to assess FRET—The process of data collection is summarized in Figure 4 and examples of the emission scan data are shown in Figure 5A. There was negligible FRET, as measured by donor quenching (0–5%) for mixtures of fluorescent calpain and cL-ABC-D1 calpastatin in the absence of calcium. Addition of calcium to these mixtures produced FRET measured as the relative increase in Em_{Acc}/Em_{Don} (Figure 5 A and B, Table 1) for cL-ABC-D1 calpastatin with unique cys- N terminal to the conserved A peptide (Ser₁₄₂Cys, Ser₁₄₈Cys, or Ser₁₅₁Cys) or C terminal to the conserved C peptide (Ala₂₄₆Cys). With calpain Cys₁₀₅Ala-eGFP (0.3–0.4μM) and AlexaFluor546 (AF546) labelled cL-ABC-D1-calpastatin (typically ~1.5μM, but varied 0.5–5 μM) the Em_{Acc}/Em_{Don} increased ~2 fold ($99.9 \pm 19.2\%$) upon addition of calcium (>0.5mM, Figure 5B and typically ~3 mM, Table 1). Assays with cL-ABC-D1-C₂₂₃ –AF546, i.e. a cys residue N-terminal to the induced C-peptide helix, yielded only a ~30% increase in Em_{Acc}/Em_{Don} upon calcium addition. In contrast to peptides with unique cysteines, the AlexaFluor treated peptide lacking cysteine showed <10% increase in the Em_{Acc}/Em_{Don} in response to calcium. Thus the FRET signal observed for peptides with unique cys residues was 4–15 fold greater (Table 1) than the nonspecifically labeled material. Binding of calpain Cys₁₀₅Ala-LumioGreen and cL-ABC-D1 calpastatin labeled with either TMR or AF546 at residues 142,148 or 151 produced an increase in $Em_{Acc}/Em_{Don} = 330 \pm 50\%$ (n=6) relative to ~20% increase observed with thiol-free peptide (a ~16.5 fold signal:noise). Although the FRET response was more robust using LumioGreen labeled calpain, most assays were conducted with calpain Cys₁₀₅Ala-eGFP for cost and convenience.

Similar FRET efficiencies were calculated for cL-ABC-D1 labelled with AF546 at C₁₅₁ (N-terminal to the A peptide) or C₂₄₆ (C-terminal to the C peptide) (Table 1) suggesting that both positions are equally effective as FRET acceptors and are expected to be ~equidistant from the C terminal eGFP on calpain based on the structure from Todd et al.[9]. In contrast FRET measurements with cL-ABC-D1 labelled with AF546 at C₂₂₃ demonstrated lower efficiency (~13%) (Table 1) consistent with the orientation of the C peptide and placing it further from the C terminus of calpain-dIV-linked eGFP.

Addition of EGTA reverses the calcium dependent, calpastatin-calpain interaction and as predicted resulted in relief of donor quenching and attenuation of FRET. The EGTA dependent decrease in Em_{Acc}/Em_{Don} (~37% for cL-ABC-D1; corresponding to a 75–90% reversal of the calcium induced increase) further demonstrates the calcium dependence of the protein-protein interaction needed for FRET. The reversibility of the response also suggests that donor quenching is not a result of calcium dependent aggregation of calpain, a process that would be irreversible. Assays with nonspecifically labeled, thiol free calpastatin peptides showed no measurable decrease of Em_{Acc}/Em_{Don} in response to the addition of EGTA and further supports the conclusion that the uniquely labeled cys residues are responsible for the observed FRET.

Similar experiments with the N-terminal fragment of calpastatin domain 1 (cL-AtB-D1, Figure 1C: Ser₁₄₈Cys-AF546 or Ser₁₅₁Cys-AF546) showed a somewhat less robust calcium dependent increase in FRET relative to the complete cL-ABC-D1 (Figure 5 and Table 1). cL-AtB-D1-C₁₅₁-AF546 also displayed a lower and more variable efficiency of FRET likely a result of its being more weakly bound (see below). In contrast to cL-AtB-D1, the C-terminal calpastatin-D1 fragment that only contained the conserved C peptide (C-D1, Figure 1D) with modified Cys residues at 223 or 241 or 246 or 256 failed to demonstrate any calcium dependent

FRET with either fluorogenic form of calpain; a $7.6 \pm 5.0\%$ increase in Em_{Acc}/Em_{Don} , similar to that observed with non-specifically labeled peptides (Table 1). Lack of FRET for C-D1 labeled at cys₂₄₆, is of particular note because strong FRET is observed using cL-ABC-D1 modified at that residue. To address the possibility that in the absence of the complete calpastatin domain, calcium-calpain is less stable and more prone to aggregate, assays of the fluorogenic C-D1 calpastatin were also conducted in the presence of unmodified cL-AtB-D1. The added calpastatin fragment did not rescue a FRET response from CD-1 C₂₄₆AF546 nor did the converse experiment (addition of the unlabeled C-D1 calpastatin to assays utilizing the fluorogenic cL-AtB-D1 restore the magnitude of the response to more closely resemble that of cL-ABC-D1 (data not shown).

In order to begin to assess the potential utility of a calpastatin based reagent for detecting active calpain in cells, binding assays were also conducted using a crude lysate from *E. coli* induced to express calpain Cys₁₀₅Ala-eGFP. *E. coli* expressed enzyme was highly advantageous as this lysate lacks endogenous, non-fluorogenic calpain and calpastatin that would be expected to compete for binding with reagents. Importantly the high affinity and specific binding between calpain and calpastatin is demonstrable in the presence of ~5 mg/ml assorted proteins although the calcium dependent increase in Em_{Acc}/Em_{Don} was less robust (35–50%) than in the assays with more highly purified donor (~99%). Addition of a commonly used and highly preferred calpain-2 substrate, α -casein (20 μ M) did not alter the FRET response in vitro.

3.2 Disrupting FRET between calpain Cys₁₀₅Ala-eGFP- and calpastatin-fragments

The addition of non-fluorogenic calpains, such as calpain-2 Cys₁₀₅Ala or active calpain-1 (in the presence of small molecule inhibitors to prevent proteolysis), was expected to compete for binding of the labeled calpastatin fragments and therefore reduce the FRET response. With unmodified calpain-2 or calpain-1 present at ~0.35 μ M (a concentration approximately equivalent to the donor calpain-2-eGFP in the assay) inhibition of FRET was ~50% and demonstrates that, as expected the calcium-bound, unmodified calpain-2 and calpain-1 also form complexes with the fluorogenic calpastatin fragment. Surprisingly, addition of unlabeled cL-ABC-D1 calpastatin to the complex of calpain Cys₁₀₅Ala-eGFP and cL-ABC-D1-AF546 did not reduce FRET significantly indicating a slow off-rate (or slow exchange rate) for the bound calpastatin fragment. This interpretation is supported by recent measurements of the kinetics of binding between immobilized calpastatin domains and calpain-2 [37]. Therefore it was necessary to mix fluorogenic and unlabeled calpastatin peptides with calpain prior to the addition of calcium in order to assess any decrease in FRET caused by the unmodified, competing peptide. As expected dilution of each fluorogenic calpastatin peptide with its unlabelled parent peptide attenuated the FRET signal in a concentration dependent manner (Figure 6A, 6B). The relative binding constants for unlabelled peptide in comparison with its AlexaFluor modified forms (at Cys 246 or Cys148 for cL-ABC-D1 and cL-AtB-D1-Cys-151 or 148) were approximately 1 (1.37 ± 0.23 or 1.25 ± 0.13 respectively) based on the data from the competition assays. This confirmed that there is no significant difference in binding between the chemically modified and unmodified calpastatin peptides. Fluorogenic cL-AtB-D1 however was more readily displaced by cL-ABC-D1 (Figure 6C) indicating its relatively weaker interaction with calpain; a factor that could also contribute to its decreased inhibitory activity. In fact, weaker binding may be advantageous for the sensitivity of competitive binding assays to measure of calpastatin (or calpain) in crude samples. Because calpastatin concentration relates to meat quality and tenderness, there have been previous attempts to use a FRET-based assay, utilizing fluorogenically labeled antibodies, to measure calpastatin in meat extracts [38]. To determine if the binding assay described here could be used to measure calpastatin in a crude sample, bovine heart meat was processed to provide a calpain-depleted, heat-stable fraction as a crude calpastatin sample. Addition of the crude calpastatin fraction

produced a concentration dependent loss of FRET (Figure 7) and provides a measure of the relative calpastatin proteins/fragments present in the sample.

Discussion

Much prior work with calpain and calpastatin including biochemical assays of its functional domains [5,34,35,39], binding studies with synthetic peptide mimics of conserved regions of calpastatin and isolated domains of calpain [20–22,25,40,41], and the structure of the C-peptide bound to dVI [9] provided key information about the significance of specific calpastatin regions or residues involved in its inhibitory activity and binding to calpain. With the aim of developing fluorescent reagents that distinguish between the calcium free (inactive) and calcium bound, catalytically-competent, conformations of calpain we exploited the existing information to chemically modify a domain of calpastatin, or its component fragments, to provide proof of concept for FRET based detection of calcium bound calpain-eGFP.

Fluorogenic modification of cL-ABC-D1 near the N-terminus (residues 142, 148,151) of the conserved A peptide (S148-G165) or C-terminal (residue 246) to the conserved C peptide (S223-C241) did not impair its inhibitory activity or binding to calpain-eGFP. The calcium dependent FRET observed between these modified calpastatin peptides and calpain-eGFP validates that the sites of interactions between calpastatin mimic peptides (19–27 residues) and isolated, and sometimes immobilized, domains IV or VI of calpain also occur in the complex formed between the calpain-2 holoenzyme (i.e. the calcium bound heterodimer) and a complete, functional domain of calpastatin in solution. The possibility that alternative binding interactions may occur when target peptides are presented within the context of a larger protein, as was recently demonstrated for several calmodulin–target peptide complexes [42], necessitates such experimental validation of interactions predicted from studies with small synthetic peptides. The lack of FRET between either cL-AtB-D1 or cL-ABC-D1 calpastatin and calpain-eGFP in the absence of calcium suggests that despite the potential of pre-formed primary contact sites present in calpastatin [43] stable binding does not occur in the absence of calcium. According to modeling efforts by Todd et al [9] either orientation of the A peptide was compatible with its predicted binding groove on dIV. From the assays reported here, it appears that the N- terminus of the A subdomain and C terminus of the C peptide are similar distances from the C terminus of calpain based on their FRET efficiency, thus establishing the preferred orientation of the A peptide. Also consistent with the tripartite calpastatin binding model, modification of cL-ABC-D1C₂₂₃, a position N terminal to the conserved C peptide, yields a lower and less efficient FRET response.

Recently published NMR studies utilizing isotopically labeled calpastatin-domain 1 (a peptide very similar to cL-ABC-D1) in the presence of calpain and calcium further verify calpastatin's binding interaction with calpain-2 in solution, although those studies were performed at lower pH (pH 6.18), 15 mM NaCl and required mM calpastatin [44]. Importantly Kiss et al further define the induced helical regions and identified tight contact sites within the calpain-calpastatin complex at calpastatin's A (A₁₅₆, D₁₅₈, T₁₆₃) and C (D₂₂₉, S₂₂₇ and F₂₃₉) peptides [44]. The residues we modified (i.e. 142, 148, 151, 223, 246) are in regions shown to retain their intrinsically unstructured characteristics even when complexed with calpain; a finding consistent with our results that modification of any one of those residues does not impair binding.

The calcium sensitivity of the FRET response resulting from complex formation with cL-ABC-D1 and cL-AtB-D1 is also consistent with independent measures of the calcium dependence (~250–350 μ M for half-maximal activity) of calpain activity reported for the catalytically competent form of this recombinant enzyme [45]. Although the measured affinities for individual synthetic mimics of the calpastatin A, B and C peptides are similar to each other

and to that reported for a complete functional domain [20], competition experiments (Figure 6C) show that the construct truncated within the B peptide is more easily displaced than the complete domain. This argues that the multi-site binding potential of a complete inhibitory domain retains higher avidity than the bi-partite binding of the A-peptide plus key, B peptide residues. In contrast to a report that the 19mer mimic of calpastatin's C peptide reduces calpain's calcium requirement [46] addition of the expressed C-D1 peptide containing the C peptide-19mer in the context of an 80 residue peptide (at 1:1 or 5:1 relative molar concentration) had no significant impact on calcium dependence of binding of the non-overlapping cL-AtB-D1 fragment.

The failure of C-D1 to generate calcium dependent FRET, even when fluorophore was bound to a position effective in generating FRET within the complete cL-ABC-D-1, suggests that either the single C peptide binding interaction is not sufficiently stabilized in solution to produce FRET or it may indicate that the assay conditions favor dissociation of the truncated small subunit (dVI) (a long time controversy in the calpain field, [5]) and thus the C peptide is no longer proximal to the C-terminal label on calpain. This interpretation is consistent with the preference of the subdomain C peptide for domain VI, although the C peptide can also bind the isolated dIV with ~10 fold lower affinity [24] and the A peptide can bind dVI [20,24]. The C terminal, vector-encoded, 18 residues are unlikely to interfere with binding or FRET as its composition and position would be identical in cL-ABC-D1 and the C-D1. Future studies are needed to determine if extending C-D1 to include key binding regions of the B peptide (e.g. **LGKREVTIPP-KYRELL**) is sufficient to stabilize binding of the C-D1 fragment. It may also be informative to determine if deletion of these same residues destabilizes the FRET signal obtained with cL-AtB-D1. The covalent linkage of calpastatin's subdomains is apparently essential because adding the cL-AtB D1 peptide (1:1 or at a 5:1 excess) to the labeled C-peptide showed no rescue of the FRET signal. Theoretically, binding of the A and C peptides as amphiphilic helices to dIV and dVI respectively would place them close together (~6.2 nm) suggesting another possible approach for designing a FRET probe. However FRET is observed between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) flanking a functional, unstructured and flexible calpastatin domain in the absence of enzyme binding [14] and therefore this strategy was not pursued.

Deletion mutagenesis studies of calpastatin and enzyme assays with synthetic B-peptide mimics (19–27 residues) [21,22,40,41] demonstrate that this peptide is both necessary and sufficient to inhibit calpain. More recent studies determined the importance of L₁₉₄ and I₂₀₁ in key hydrophobic interactions that mediate high affinity inhibition of calpain-1 [21,22]. Mixtures of B peptide fragments, split between L₁₉₄G and TI₂₀₁P, were less effective inhibitors suggesting that their covalent linkage is critical for optimal function [21,22]. Because cL-AtB-D1 retained the L₁₉₄G---TI₂₀₁P sequence, it was not too surprising that it also inhibited calpain albeit less efficiently than the intact cL-ABC-D1. Stable binding of cL-AtB-D1 is expected to occur via the tight binding of the A peptide and thus apparently allows this minimal fragment of the B sequence to have some inhibitory effect. This result is consistent with earlier work showing that disruption of the A peptide helix was more effective in attenuating inhibitory activity [23] than disruption of the C peptide helix for a calpastatin fragment missing TIPPKYR of the B peptide. Interestingly, early (and often overlooked) studies demonstrated that deletion of the B peptide core TIPPKYR or G₁₉₅KREVTIPPKYRELL₂₀₉ did not completely eliminate inhibition by a calpastatin domain [23] though we know of no evidence suggesting that either the A or C peptides alone or together can themselves inhibit calpain. Increasing evidence for multiple binding interactions between calpain and calpastatin coupled with calpastatin's susceptibility to a variety of proteases provides a potential mechanism to finely tune enzyme activity and/or to modulate substrate specificity. To our knowledge this suggestion has not been previously made or investigated.

To be applicable to a more cellular setting, specificity of a calpastatin based reagent must be demonstrated in the background of a complex mixture of proteins. Success of this assay with recombinant calpain Cys₁₀₅Ala-eGFP in crude lysates of E coli BL21DE3 and the observed lack of interference from the presence of a preferred in vitro substrate (casein) provide initial steps towards this goal. As expected unlabeled active calpain-1 competes for calpastatin binding and a calpain-depleted, heat stable extract of muscle (heart) that contains calpastatin did compete for binding of cL-AtB-D1. Thus the assay can provide a relative measure of calpain or calpastatin. The likelihood of multiple forms of calpastatin in a crude extract may further complicate its quantification but quantification of calpastatin as cL-ABC-D1 'equivalents' could be used to account for the mixtures of full length, splice variants and assorted fragments of calpastatin that are expected to compete with varying affinities in the binding assay. Experiments with a variety of mammalian cells and extracts will be difficult due to the presence of both endogenous calpains and calpastatin and the likelihood that calpain regulation will be context dependent. Susceptibility of calpastatin to a variety of proteases, a likely hazard of its unstructured nature provides a further complication of applying this FRET based assay to in situ conditions. Therefore additional approaches for detecting the active enzyme, such as activity dependent labeling strategies (e.g. self labeling protein tags similar to those developed for ubiquitin ligase and O⁶-alkylguanine-DNA alkyltransferase) and activity based profiling reagents [47–51] must also be pursued.

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A. Schematic of calpastatin domain organization: **B. cL-ABC- Domain-1 (cL-ABC-D1) Calpastatin**

MV₁₁₄AGITAI₁₂₀SGKPGDKKKE₁₃₀KKSLTPAVPV₁₄₀ES₁₄₂KPKDKP_{S148}GKS₁₅₁GMDAALDD
L₁₆₀IDTLGGPEETEEENTTYTGPEVS DPMSSTY₁₉₀IEELGKREVT₂₀₀IPPKYRELLA₂₁₀
 KKEGITGPPA₂₂₀DSSKPIGPDD₂₃₀AIDALSSDFTC₂₄₁GSPTA₂₄₆AGKKTEKEESTEVL₂₆₀
 KAQSAGTVRS₂₇₀ EFELHKLAAALEHHHHHH

Variants: 1) C₂₄₁S; 2) S₁₄₂C-C₂₄₁S; 3) S₁₄₈C-C₂₄₁S; 4) S₁₅₁C-C₂₄₁S; 5) C₂₄₁S -A₂₄₆C;
 6)C₂₄₁S-S₂₂₃C

C. cL-A-tB-Domain-1 (cL-A-tB-D1) Calpastatin

MV₁₁₄AGITAI₁₂₀SGKPGDKKKE₁₃₀KKSLTPAVPVE_{S142}KPKDKP_{S148}GKS₁₅₁GMDAALDDL₁₆₀
IDTLGGPEET₁₇₀EEENTTYTG₁₈₀EVS DPMSSTY₁₉₀IEELGKREVT₂₀₀IP
 EFELHKLAAALEHHHHHH

Variants: 1) S₁₄₂C; 2) S₁₄₈C; 3) S₁₅₁C

D. C peptide - Domain-1 (C-D1) Calpastatin

MA₂₁₀KKEGITGPPA₂₂₀DSSKPIGPDD₂₃₀AIDALSSDFTC₂₄₁GSPTA₂₄₆AGKKTEKEEST
 EVLKAQSAGTVRS₂₇₀ EFELHKLAAALEHHHHHH

Variants: 1) C₂₄₁S; 2) C₂₄₁S -A₂₄₆C; 3) S₂₂₃C -C₂₄₁S; 4) C₂₄₁S -S₂₅₆C

Figure 1. Domain structure of calpastatin and sequence of domain-1 constructs

1A. Calpastatin (human) is a 708 amino acid residue protein with an amino-terminal leader sequence (L-domain) and four inhibitory domains (I–IV). Each inhibitory domain is ~140 residues and contains three highly conserved sequences designated as subdomains or peptides A, B, and C. The A and C peptides (~20 residues) are similar to each other and flank the unique, essential inhibitory B peptide (~26 residues).

1B–D. The fragments of calpastatin used for binding assays. The peptide defined by the sequence (given as one letter code) in Panel B contains the carboxy terminus (34 residues) of the L domain (cL) and the complete first inhibitory domain (ABC) of calpastatin (human) and is designated cL-ABC-D1. In each panel the A and C peptide sequences are underlined and

the B peptide residues are boxed. The induced helical regions within the A and C conserved peptides are indicated with bold italics in 1B. The naturally occurring cys241 and other residues mutated to encode cys in individual constructs are numbered and bold. An initiator Met (M) and C terminal extension (double underlined) including a hexa-his (H6) affinity tag were encoded by the vector. Panel C defines the amino terminal fragment of cL-ABC-D1 that truncated the B peptide and is designated cL-AtB-D1. Panel D shows the sequence of the C-terminal fragment designated as C-D1.

Figure 2. Structure predicts that fluorigenically modified calpain and calpastatin may produce FRET upon binding

The structure of the homodimer of dVI bound to calcium and a C-peptide (19-residues) of calpastatin was determined by Todd et al [9] (pdb file 1NX1). Panel 2A was created using that pdb file to provide a model for the dIV-dVI heterodimer, with one dVI chain (yellow) to represent dIV and the other (orange) dVI. The bound calpastatin- C peptides would similarly represent the calpastatin A peptide (position of cyan peptide) and C-peptide (purple). Red spheres designate the N terminal residue of the cyan “A” peptide and the C terminal residue of the “C” peptide (purple) to highlight the relationship between them (shown by longer black line) and between the N-terminus of the bound calpastatin A peptide and the C terminus of calpain (green spheres; shorter black line) The eight calcium ions are shown in magenta. Panel 2B shows the calpain-2 heterodimer in the absence of calcium (pdb file 1DF0) and demonstrates the association of dIV (yellow) and dVI (orange) in the inactive enzyme in context with the core catalytic domains (dI and dII) and the C2-like dIII. Positions of the catalytic residues are indicated with magenta sticks. Ribbon cartoons shown were generated with Pymol (DeLano Scientific LLC). The schematic in Panel 2C provides an overview of the components used for the assays (left side) and the expected calcium-dependent, binding interactions between calpain-eGFP and calpastatin fragments (right side) assessed for potential FRET between the calpain-linked eGFP and fluorophore-modified residues within calpastatin-D1 or its fragments.

Figure 3. Analysis of the proteins used in binding assays by SDS-PAGE

Purified and partially purified proteins were examined by electrophoresis (9% (panel A) or 10% (Panel B) acrylamide using Tris-Tricine buffers) with denaturing conditions (SDS-PAGE). Panel A. Prior to modification proteins were visualized with Coomassie blue-amido black and imaged as described in methods. Both lanes “M” show molecular weight markers that represent 94, 67, 43, 30, 20 and 14 kDa from largest to smallest. Lanes 1 and 2, (6 μ g and 8 μ g protein respectively) show purified cL-ABC-D1. Like most calpastatin fragments its apparent molecular weight by SDS-PAGE (~27kDa) exceeds its actual size (18.7kDa). Lanes

3–5 show purified calpain 2 (80kDa-Cys₁₀₅Ala with 21kDa small subunit); the variant containing the C terminal binding motif for Lumiogreen-cys-cys-pro-gly-cys-cys; and partially purified calpain-Cys₁₀₅Ala-eGFP; respectively with 4 µg protein per lane. The fusion protein of calpain-eGFP in lane 5 (~109kDa) is indicated as 80*. Lanes 6 and 7 (5 µg each lane) depict the purified cL-AtB-D1-Cys₁₅₁ and C-D1-Ser₂₄₁Cys₂₅₆ respectively with anomalous migration of each peptide observed: ~17kDa rather than 11.7kDa for cL-AtB-D1 and ~12kDa rather than 8.4kDa for C-D1. Results shown are representative of all proteins utilized in the studies described. Panel B shows a gel viewed for fluorescence (upper image) and by Coomassie blue-amido black staining (lower image) for AlexaFluor546 modified peptides [3.5µg (odd lanes) or 9 µg (even lanes)]. Proteins containing a unique cysteine cL-ABC-D1-Cys₂₂₃-AF546 (lanes 1,2) and cL-AtB-D1-Cys₁₄₂-AF-546 (lanes 7, 8) and their cysteine lacking counterparts (cL-ABC-D1 Cys₂₄₁Ser-lanes 3,4 and cL-AtB-D1 lanes 5,6) are shown. The approximate location of the ion front is indicated in near the bottom of the gel picture by a horizontal line. Note the absence of any fluorescence associated with the peptides lacking a thiol group (lanes 3–6) and the significant low molecular weight fluorescence that is not coincident with the peptides and is particularly prominent in the samples for peptides lacking cysteine.

Assay Design

Figure 4. Overview of data collection - assay design

The data collection process for typical experiments is shown. Data processing and calculations were performed as described in Methods.

Figure 5A

Figure 5B

Figure 5. FRET between calpain-eGFP and labeled calpastatin fragments is calcium dependent

Panel A illustrates typical emission scans from assays conducted as outlined in Figure 4. In each panel: data are shown for donor —; donor + acceptor; donor + acceptor + calcium (to 2.5 mM) ---; and subsequent addition of EGTA (15 mM excess) Thus the difference between the grey dotted line and the red dashed line illustrates quenching of donor emission at 508 nm and enhanced acceptor emission at ~570nm. The difference between the red dashed line and turquoise dot-dash line demonstrates the relief of donor quenching and loss of enhanced acceptor emission in response to EGTA. The left panel depicts data from an assay with calpain-C₁₀₅A-eGFP and cL-AtB-D1-Cys₁₅₁-AlexaFluor546. Em_{acc}/Em_{don} increased 0.2204 upon addition of calcium (a 64.4% increase) and decreased 0.1845 after addition of EGTA. The right panel depicts emission scans from an assay with calpain-C₁₀₅A-eGFP and cL-ABC-D1-Cys₂₄₆-AlexaFluor546. Em_{acc}/Em_{don} increased 0.4564 upon addition of calcium (a 70.0% increase) and decreased 0.4047 after addition of EGTA.

Panel B. Binding assays were performed with cL-ABC-D1-Cys₁₅₁-Alexafluor546 (■) or cL-AtB-Cys₁₅₁ Alexafluor546 (□) and calpain-Cys₁₀₅Ala-eGFP (0.3 μM) with calcium increased incrementally. Emissions scans were collected after each addition of calcium and the assay was terminated with addition of EGTA to 18 mM. Free calcium was calculated via WEBMAXC Standard (Maxchelator) and calcium dependent changes in Em_{Acc}/Em_{Don} were calculated by subtracting Em_{Acc}/Em_{Don} measured in the absence of calcium from Em_{Acc}/Em_{Don} at each calcium concentration. The average maximal calcium dependent change in Em_{Acc}/Em_{Don} was taken as 100% and data are expressed relative to that average maximum. Results shown are representative of similar experiments (n= 5 for cL-ABC-D1 and n= 7 for cL-AtB-D1) with a range of donor (0.25–1 μM) and acceptor (1–4 μM) concentrations and with the modified cys at residue 148, 151 or 246 of calpastatin fragments. The maximal increase in Em_{Acc}/Em_{Don}

averaged 0.4504 ± 0.1965 for cL-ABC-D1 constructs (n= 5) and 0.1691 ± 0.042 (n=7) for cL-AtB-D1.

Figure 6. Addition of unmodified calpastatin peptides decreases the FRET response

Calcium dependent FRET between calpain-Cys₁₀₅Ala-eGFP and cL-AtB-D1-Ser₁₅₁Cys-Alexafluor546 (Panels A and C) or cL-ABC-D1-Cys₂₄₁Ser-Ala₂₄₆Cys Alexafluor546 (panel B) or was measured in the absence (100% response) or presence of varying amounts of non-fluorogenic peptides with the ratio of unmodified peptide to fluorogenic peptide given (0.01:1–2.5:1) as indicated. Data are expressed relative to the maximal decrease in $E_{\text{acc}}/E_{\text{don}}$ measured after addition of 18 mM EGTA. Maximum decrease = 0.1029, 0.1845 in Panel A; 0.0718, 0.3355, 0.4047 in Panel B and 0.0963, 0.1532 in Panel C. Data plotted are the composite results from two (Panels A and C) or three (Panel B) experiments and representative of results of two additional experiments.

Figure 7. Calpastatin can be measured in a heat stable fraction of meat extract

Binding assays between calpain-Cys₁₀₅Ala-eGFP (~0.45 μM) and cL-AtB-D1-Cys₁₅₁-Alexafluor546 (~1.5 μM) were performed in the presence of 1 mg/ml added protein that contained only BSA (0 heat stable heart protein) or various amounts of a heat stable heart protein fraction that contains calpastatin (but not calpain). Data are expressed as % maximal decrease of Em_{Acc}/Em_{Don} (0.1705 and 0.1589 in these two experiments) that occurred after the addition of EGTA (18mM). Data plotted are results from two experiments and similar to results from two additional experiments.

Table 1

Summary of FRET assay results

(calpain-C105A-eGFP as donor)	
Acceptor	Increase in Em_{acc}/Em_{don}
cL-ABC-D1 ^a	99.9 ± 19.2% (n=15)
cL-ABC-D1-C ₂₂₃	34.1 ± 12.8% (n=5)
cL-AtB-D1 ^b	66.7 ± 8.4% (n=11)
C-D1	7.6 ± 5.0% (n= 9)
Cys-free cL-ABC-D1 ^c	8.4 ± 3.6% (n= 5)
Acceptor	FRET Efficiency
cL-ABC-D1	
S ₁₅₁ C-AF546	32.7 ± 2.3% (n=5)
A ₂₄₆ C-AF546	33.5 ± 0.4% (n=4)
S ₂₂₃ -C-AF546	13.2 ± 1.0% (n=4)
cL-AtB-D1	
S ₁₅₁ C-AF546	25.3 ± 4.3% (n=7)

^a composite results from peptides labelled with AF546 at either residue 142, 148, 151 or 246

^b composite results from peptides labelled with AF546 at either residue 142, 148 or 151 For both cL-ABC-D1 and cL-AtB-D1: calpain-eGFP ranged from 0.3–0.5 μM and acceptor peptides varied from 0.5–3 μM. For most data collection the acceptor (~1–1.5 μM) was 2–4 times the donor concentration.

^c Cys free- cL-AtB-D1 also demonstrated minimal calcium dependent increase (13.3 or 3.6%) in Em_{acc}/Em_{don} in two separate experiments.