ENGINEERING CALPASTATIN TO DEVELOP A SENSOR TO DETECT ACTIVE CALPAIN

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By Lisa M. Vanhooser
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Calpains, Ca\(^{2+}\)-activated cysteine proteases are essential for early embryonic development and function in signal transduction, cell adhesion, and apoptosis. Calpains also contribute to cataractogenesis, myocardial infarctions, and neurodegenerative diseases such as Alzheimer’s. The various methods currently available to demonstrate these roles do not directly identify spatial or temporal activation of calpain in cells. Therefore, a tool to detect active calpain in situ will be useful.

Calpastatin is the ubiquitous, endogenous inhibitor that specifically binds the active conformation of the conventional calpains. Calpastatin consists of four homologous domains each containing three subdomains A, B, and C. The crystal structure of a calpastatin C peptide mimic bound to the homodimer of calpain domain VI in the presence of Ca\(^{2+}\) provides a model for the structure of calpastatin subdomains A and C bound to calpain domains IV and VI (56). This structure predicts the calpain domain IV carboxy-terminus is approximately 31 Å from the bound calpastatin domain I amino-terminus and the calpastatin domain I subdomain A amino-terminus is approximately 50 Å from the calpastatin domain I subdomain C carboxy-terminus when bound to calpain. Both are distances that should allow fluorescence resonance energy
transfer (FRET) between a pair of fluorophores. These interactions provide a strategy to develop a FRET based sensor to detect active calpain.

A strategy to detect endogenous calpain using the binding of fluorophore modified calpastatin subdomain A and C peptides has not yet provided positive results. An alternative strategy utilizing the binding of calpastatin domain I to calpain 2 has successfully demonstrated FRET. Three calpastatin domain I peptides were labeled at a single distinctly located cysteine residue near the amino-terminus with maleimide conjugated tetramethylrhodamine or Alexa Fluor (AF) 546. Lumio Green specifically binds a rare tetra-cysteine motif not found in native calpain 2. Insertion of this motif or eGFP at the carboxy-terminus of calpain 2 domain IV provided two mechanisms for specific labeling of calpain to serve as donors to six acceptor fluorophore labeled calpastatin domain I peptides during in vitro binding assays. All twelve donor-calpain acceptor-calpastatin pairs demonstrated a calcium dependent increase in a ratio of acceptor emission to donor emission ($E_{\text{a}}/E_{\text{d}}$) indicating the detection of a FRET signal. The increase in $E_{\text{a}}/E_{\text{d}}$ ratio was detected in an *E. coli* crude cell lysate expressing calpain-eGFP to demonstrate the approach should also work in the complex cellular environment. As expected, calpastatin, calpain 1 and calpain 2 did compete for binding of calpastatin-AF546 to calpain-eGFP. In contrast, two calpain substrates, the cytoplasmic tail of β1 integrin and α-casein, did not exhibit competition. These results demonstrate that most substrates should not interfere with the binding signal when applied in situ, however endogenous classical calpains and calpastatin likely will interfere to some extent. Future studies aim to produce a pair of fluorogenic calpastatin subdomain peptides that bind calpain and generate FRET to allow detection of endogenous calpain in cells.
ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Dorothy Croall for allowing me the opportunity to work in her lab. Initially she had faith in my potential when others did not and offered me the opportunity to prove that I could do better.

I would also like to thank my committee Dr. Robert Cashon and Dr. Robert Gundersen. Whether at committee meetings, in class, advice in the hallway, and help analyzing data, both have been very supportive and helpful throughout the past two years.

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$C_{148S241}$-AF546 Binding to $C_{105A}$-eGFP
Chapter 1 INTRODUCTION

Calpains and Their Significance

Within the calpain family of calcium activated, neutral cysteine proteases, calpains 1 and 2 are two classical isoforms of the EF-hand subfamily (26). Physiologically, these calpains are implicated in the progression of cell cycle, long-term potentiation, cytoskeletal remodeling and apoptosis. A critical role for the classical calpains was demonstrated when disruption of the Capn4 gene was embryonically lethal to mice (66). This gene encodes the small subunit common to the heterodimer of the classical calpain isoforms 1, 2 and 9. It was later discovered that disruption of the Capn2 gene encoding the calpain 2 large subunit was also embryonically lethal in mice. These experiments demonstrated that calpain 2 is an essential calpain isoform for successful murine fetal development (14).

Additionally, calpains are implicated in cell adhesion and motility. Calpains have been identified in early integrin clusters that form the initial cellular adhesion contacts of bovine aortic endothelial cells (2, 3). Calpain activation has been identified in these early integrin clusters upstream of Rac activation leading to formation of filopodia, extension of lamellipodia and restructuring of the early integrin clusters into focal complexes (17). Furthermore, calpain activity has been associated upstream to RhoA activation leading to the formation of stress fibers and focal adhesions, the long term integrin adhesions. Together these and other data support a role for calpain in integrin mediated cell adhesion and motility.

Calpains have also been linked to pathological roles in conditions such as muscular dystrophy, cataractogenesis, multiple sclerosis, cardiac ischemia, and neurodegenerative diseases (5, 23). Of particular interest is calpain's role in Alzheimer's Disease. Aggregation of the
amyloid precursor protein (APP) has been associated with the activation of calpain prior to apoptosis (5, 32). Presenilin 1 forms the active site of the multiprotein complex γ-secretase that is responsible for the proteolytic processing of the APP (29). At least in early onset familial Alzheimer's disease, there is evidence to support that mutations in the APP binding site of presenilin 1 increases cells' susceptibility to apoptosis possibly through phosphoinositide stimulated increases in intracellular calcium (6). This increase in calcium may be the mechanism by which calpain is activated in Alzheimer's disease.

**Current Methods for Detecting Active Calpain**

The importance of ascertaining calpains' roles in cellular processes led to attempts to identify its subcellular localization when active. Calpains 1 and 2 are also known as μ and m calpain in reference to their respective in vitro calcium requirements; 3-50 μM for calpain 1 and 0.4-0.8 mM for calpain 2 (18). The submicromolar calcium levels in the global cellular environment are not sufficient for activation (23) suggesting additional factors are required for activation in vivo. Localization near calcium channels and the endoplasmic reticulum may position calpains near areas with short term increases in calcium sufficient for activation. Two regions common in both calpains 1 and 2 have the potential to bind membranes. Many identified calpain substrates including talin, vimentin, actin, fodrin, and β integrins are in fact membrane associated proteins (9). In vitro studies monitoring calpain 1 cleavage of fodrin demonstrated lower calcium requirements in the presence of phosphatidylinerine and phosphatidylinositol (48). Based on this evidence calpains may have the ability to bind membranes for localization as well as activation.

The various combinations of methods utilized thus far do not directly identify active calpain's localization. These methods include in vitro assays to identify proteins as calpain
substrates, in situ colocalization of calpain with these substrates and other proteins, and in situ calpain overexpression or inhibition. Proteolysis of calpain substrates in cell lysates is used to demonstrate a role for active calpain during cell adhesion, apoptosis and Alzheimer's disease (3, 22, 30, 32, 35). Calpain substrates are initially identified through cleavage of purified proteins in vitro, proteolysis of these calpain substrates in whole cell lysates is then demonstrated on western blots (30). Cleavage of fluorogenic peptides is also used to identify calpain activity in cells. This may demonstrate calpain was active but not where within the cell. Also, it is not certain that cleavage of calpain substrates or peptides in cells is directly diagnostic for calpain activity. The mechanism of calpain's specific recognition of substrates has yet to be elucidated. Calpains' prefer solvent exposed unstructured linker regions on large proteins rather than small peptides for substrate cleavage sites (9, 55, 58). Furthermore, calpain substrates such as α-spectrin and tau can be cleaved by other proteases including caspase-3 leaving a similar sized cleavage product (60). Thus cleavage of calpain substrates may not be exclusive to calpain proteases in vivo. In situ overexpression or inhibition of calpain is used to reinforce active calpain's effect on cells during potential physiologic and pathologic roles (3, 30). Colocalization of calpains with cleaved substrate (e.g. cleaved spectrin) and proteins that serve as markers (ER marker calnexin, Golgi marker Rab6, cell membrane phospholipid PIP₂ and adhesion transmembrane protein β₃ integrin) in cells is used to demonstrate active calpain's localization (2, 3, 22). Calpains are visualized using either a polyclonal antibody raised against a peptide mimic for the calpain 1 amino-terminal residues post proteolytic cleavage or a monoclonal antibody for calpain (47, 48). The polyclonal antibody was shown to be selective for native autolyzed calpain 1 but not calpain 2. It does not appear that available antibodies that detect autolyzed calpain 2 have been used in cells. As discussed above, spectrin is also cleaved by caspase-3 and is not exclusively diagnostic for calpain activity. The insufficient selectivity of the antibodies used diminishes direct identification of active calpain's localization in cells. Overexpression of calpain in cells does give an indication of calpain's potential effects yet the unphysiological levels of the protease may
yield exaggerated or harmful effects on cells. In situ inhibition of calpain generally uses small, cell permeable inhibitors such as carbobenzoxy-valinyl-phenylalaninal MDL 28170 (MDL), Calpain Inhibitor I N-Acetyl-Leu-Leu-Nle (ALLN), or calpeptin (2, 31, 61). These inhibitors also inhibit a tyrosine phosphatase (calpeptin), cathepsins B and L (ALLN, MDL), the proteasome and other neutral cysteine proteases (ALLN) complicating the correlation of these results to calpain’s role in cells (21, 37, 39, 50). These methods do not directly identify spatial or temporal activation of calpains in cells, a tool to accomplish this would be a useful aid in determining calpain’s role in cell signaling and disease.

**Calpastatin, the Endogenous Inhibitor of Calpains**

Calpains natural endogenous inhibitor, calpastatin, most specifically inhibits the classical calpains. Calpastatin reversibly binds the classical calpains in the presence of calcium, thus it specifically binds and inhibits calpains active conformation (41). It is estimated to be less than ~80 kDa intrinsically unstructured protein (IUP) that migrates anomalously at ~110 kDa by SDS-PAGE (24). Calpastatin is made up of five domains (figure 1.1). The function of the first domain, the L domain, or leader sequence is relatively unknown but has been suggested to regulate L-type Ca\(^{2+}\) channels (40, 42). The remaining four domains (I-IV) show 20-30% homology to each other and are each capable of inhibiting calpain with affinities in the order of I>IV>III>II (18, 27, 64). Calpastatin domains I-IV each contain three subdomains known as A, B, and C. Subdomain B is the sole requirement for inhibition. Subdomains A and C while not inhibitory, tightly bind the classical calpains. The tight binding of subdomains A and C and the calcium dependent inhibition by subdomain B work together to form the most specific inhibitor for the active classical calpains (55).
Figure 1.1  
*Diagram of Full Length Calpastatin*

Full length human calpastatin including the amino-terminal leader sequence and the four inhibitory domains (I-IV) is 708 amino acid residues. The four ~140 residue imperfect repeats contain three more highly conserved regions designated subdomains A, B, and C (24). The ~20 residue subdomains A and C flank the inhibitory ~26 residue subdomain B (55).

**Interaction Between Calpain and Calpastatin**

Solving the calcium free structure of inactive calpain 2 aided in understanding the classical calpains. Calpains consist of a novel large subunit and in the case of the classical calpains heterodimerize with a common small subunit. The large subunit contains domains I-IV while the small subunit consists of domains V and VI (figure. 1.2). Domains I and II are similar to the papain like cysteine proteases and together form the active site cleft containing the catalytic triad Cys105, His262, and Asn286. The inactive calcium free crystal structure of calpain 2 shows this triad as unassembled. It is hypothesized that a conformational shift in response to calcium binding alters calpain to its active conformation and assembles the active site. Calpain domain III is a β-sandwich C2-like domain. C2 domains have been demonstrated to bind up to three calcium molecules. These calcium bind and neutralize the negatively charged aspartate residues in the acidic loops to aid in phospholipid binding (20). Domain V of the small subunit is a glycine rich hydrophobic domain that contains a segment that forms an oblique-oriented α-helix (12). These helices are amphipathic with a hydrophobic gradient that causes shallow embedding in membranes. Although this region is removed during autoproteolysis (10, 36) it may aid in localization prior to activation. Domain IV of the large subunit and domain VI of the small
Figure 1.2  *Ribbon Diagram of the Calcium Free Recombinant Rat Calpain 2 Heterodimer*

The catalytic subunit consists of the amino-terminal helix (red), domain I (orange), domain II (yellow), domain III (green), the transducer region (blue), and domain IV (cyan). Domain V is not resolved by X-ray crystallography. The truncated small subunit shows domain VI (magenta). Domains I and II together form the active site cleft containing the unassembled catalytic triad Cys105, His262, and Asn286. Diagram created via Pymol (DeLano Scientific LLC) using accession number 1U5I from structure resolved by Hosfield et al. (25).
subunit are homologous penta-EF hand domains. EF-hands are helix-loop-helix motifs that potentially bind one calcium molecule per loop when in the open form. The first four EF-hands of domain VI cooperatively bind three to four calcium molecules (56). EF-hands typically function in pairs. Thus the odd fifth EF-hand of domains IV and VI bind forming the major contacts between the two subunits of the heterodimer. This heterodimeric calpain structure is important for the binding of a calpastatin domain.

Binding studies demonstrated that subdomain A of calpastatin binds domain IV of calpain’s large subunit (64) and subdomain C binds domain VI of calpain’s small subunit (42). CD spectra of synthetic nineteen residue peptides that mimic calpastatin domain I subdomain A and C indicated the peptides were not completely unstructured but “structurally primed” (42). This means they contain transient, unstable structure that readily transitions to full structure upon binding calpain. Subdomain A contains acidic residues mixed with hydrophobic residues on a 3-4 period. Although calpastatin is intrinsically unstructured, this suggests subdomain A forms an amphipathic α-helix (64). The similarity between subdomains A and C implies they each bind calpain as an induced amphipathic α-helix (42).

The crystal structure of the calcium bound calpain domain VI homodimer in the presence of a subdomain C peptide mimic shows subdomain C bound in two hydrophobic pockets (figure 1.3). These pockets are made up of helices E1, F1, and F2E3 of the first and part of the second and third EF hands in calpain domain VI. Specifically, calpastatin Leu_{235} and Phe_{239} are the key residues that bury into the two hydrophobic pockets exposed upon the activating conformational shift in calpain (figure 1.4). The smaller pocket opens due to a shift in calpain domain VI Leu_{102} and Trp_{166}. Calpastatin Phe_{239} forms van der Waals interactions with calpain His_{129} and Gln_{173}. The second larger hydrophobic pocket opens upon a shift in Gln_{173} while His_{129} binds calpastatin Ser_{236}. Calpain domain VI Trp_{166} ends up sandwiched between calpastatin
Figure 1.3  
**Ribbon Diagram of Calpain Domain VI Homodimer Bound by Calpastatin C Peptide Mimic in the Presence of Calcium**

The penta-EF hand domain VI of the porcine calpain small subunit (green and cyan) forms a homodimer in the presence and absence of calcium. Each calpain domain is bound by four calcium molecules (yellow spheres) and one 19 residue synthetic peptide mimic of calpastatin domain I subdomain C (red and purple). Diagram created via Pymol (DeLano Scientific LLC) using accession number 1NX1 from structure resolved by Todd et al. (56).
Figure 1.4  Binding Interactions of Calpastatin C Peptide Mimic with Calpain Domain VI
The specific amino acid residues of calpastatin subdomain C based synthetic peptide (red) contacting calpain domain VI (blue) in the presence of calcium. A) Calpain Gln173 shifts opening a large hydrophobic pocket where calpastatin Phe239 binds. Calpastatin Phe239 forms van der Waals interactions with calpain Gln173 and His129 while calpastatin Ser236 interacts with calpain His129. Calpain Leu102, Leu106, and Phe162 form a small hydrophobic pocket where calpastatin Leu235 binds. B) Calpastatin residues Phe239 and Leu235 bound in the two hydrophobic pockets on calpain, sandwich the calpain residue Trp166. Diagram created via Pymol (DeLano Scientific LLC) using accession number 1NX1 from structure resolved by Todd et al. (56).
Leu$_{235}$ and Phe$_{610}$. Thus Leu$_{235}$, Ser$_{236}$, and Phe$_{239}$ of calpastatin subdomain C are the residues responsible for the major contacts to calpain domain VI. The homology between calpastatin subdomains A and C as well as calpain domains IV and VI allows this crystal structure to serve as a model for the structure of calpastatin domain I subdomains A and C bound to calpain domains IV and VI.

**Rationale and Design of Sensors to Detect Active Calpain**

The calpain-calpastatin binding interaction provided a basis for developing a sensor to detect active calpain utilizing fluorescence resonance energy transfer (FRET). FRET is the measure of energy transfer from a donor fluorophore to an acceptor fluorophore (33). In order for energy transfer to occur there must be 1) spectral overlap, 2) close proximity/location, and 3) proper orientation between a fluorophore pair. The pair of fluorophores must exhibit an overlap of the donor fluorophore’s emission spectra and the acceptor fluorophore’s excitation spectra. Upon excitation of the donor fluorophore, an acceptor fluorophore within 60 to 100 Å will absorb the energy from the donor. The distance at which a fluorophore pair demonstrates 50% transfer efficiency is known as the Förster radius ($R_0$). The transfer of energy generates a shift in emission from primarily donor emission to acceptor emission at a longer wavelength. The fluorophore pair must also be in a parallel orientation to allow this energy transfer. A perpendicular orientation results in an orientation factor ($k^2$) of zero, which would produce a zero rate of transfer between a fluorophore pair (33).

The representative structure of calpastatin subdomains A and C bound to calpain domains IV and VI predicts the carboxy-terminus of calpain domain IV and the amino-terminus of calpastatin domain I are approximately 31 Å apart (figure 1.3). Additionally, the amino-terminus of subdomain A and carboxy-terminus of subdomain C are predicted to be approximately 50 Å
apart when bound to calpain. Both of these distances should allow FRET between a fluorophore pair. These predicted distances were the basis for two different designs of a FRET based sensor to detect active calpain. The first design places the donor fluorophores FlAsH (Lumio Green) and eGFP at the carboxy-terminus of the large subunit of calpain 2 and the acceptor fluorophores tetramethylrhodamine (TMR) and Alexa Fluor 546 (AF546) at the amino-terminus of calpastatin domain I. Calcium dependent binding of acceptor-calpastatin domain I to donor-calpain 2 is predicted to bring the fluorophore pair within ~31 Å to generate FRET. The second design places the donor fluorophore Alexa Fluor 568 (AF568) at the carboxy-terminus of a calpastatin domain I subdomain C peptide and the acceptor fluorophore Alexa Fluor 647 (AF647) at the amino-terminus of a calpastatin domain I subdomain A peptide. In this design calcium induced binding of the fluorophore modified calpastatin peptides to calpain is predicted to bring the fluorophore pairs within ~50 Å. The closer distance of the fluorophore pair in the first design is advantageous for producing a more robust FRET signal. The second design, however has the potential to detect endogenous calpain. Exploitation of the calpastatin-calpain binding interaction allows both designs to distinguish the active conformation of the classical calpains from inactive.
Chapter 2  MATERIALS AND METHODS

Materials

Tetramethylrhodamine-5-maleimide, Alexa Fluor 546-5-maleimide, Alexa Fluor 568-5-maleimide, Alexa Fluor 647-2-maleimide were from Molecular Probes/Invitrogen. Lumio Green (FlAsH) was from Invitrogen. Quik Change XL II Site Directed Mutagenesis Kit and components were from Stratagene. Human calpastatin plND(SP1)/V5-His C was a gift from Dr. Masatoshi Maki (Nagoya University, Japan) and Dr. Ronald Mellgren (Medical College of Ohio) (24, 34). pBlueScript (pBS) and pET24a plasmids were from Novagen. The pEGFP plasmid was from Clontech. Plasmids containing cDNA encoding rat calpain 2 Cl05A in pET24 and truncated rat small subunit in pACpET were from Dr. John Elce (Queens University, Ontario). Porcine erythrocyte calpain 1 was purchased from Calbiochem. Restriction enzymes were from New England Biolabs or Invitrogen (NotI and HindIII). T4 DNA ligase was from Promega. Ethane-dithiol and most other reagents were from Sigma-Aldrich.

Subcloning and Mutagenesis of Calpastatin Domain I

The full length human calpastatin sequence was amplified from the human calpastatin plND(SP1)/V5-His C plasmid by PCR using a primer pair that flanked the EcoRI site of the vector (table 2.1, #1). The full length human calpastatin polymerase chain reaction (PCR) product was digested with EcoRI and inserted into the EcoRI site of pBS (figure 2.1 A). The human calpastatin pBS plasmid was transformed into XL1Blue competent cells. Calpastatin Domain I (encoding Val114 to Ser270 as numbered in whole calpastatin) was amplified by PCR from calpastatin cDNA in pBS using primers to add a 5’ Ndel site and 3’ EcoRI site flanking the calpastatin domain I sequence (table 2.1, #2). This PCR product was digested with Ndel and EcoRI and ligated into pET24a at the Ndel and EcoRI sites. Four base pairs of the pET24 Sall
### Table 2.1  Primers for Calpastatin Subcloning and Mutagenesis

<table>
<thead>
<tr>
<th>Primer Sequences</th>
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<td><strong>Amplification</strong></td>
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</tr>
<tr>
<td>5'-GCTCGGATCCACTAGTCCAG-3'</td>
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</tr>
<tr>
<td>5'-GCCACTGTGCTGGATATCTG-3'</td>
<td>2</td>
</tr>
<tr>
<td>5'-CGATACCGGCTGATATQGGTTGCTGATCTGACTGCAATATC-3'</td>
<td>3</td>
</tr>
<tr>
<td>5'-GCCCTGAGAATTCTCAGCTTCCACAAAGGC-3'</td>
<td>4</td>
</tr>
<tr>
<td>5'-TCATCTGACTTCACCAGTGGGTCGCCTACAG-3'</td>
<td>5</td>
</tr>
<tr>
<td>5'-GCTGTGCGAGAGGATAAACCCGATAAACCCATTG-3'</td>
<td>6</td>
</tr>
<tr>
<td>5'-TCTAAACCCGATAGACCATCAGGATGATGCTTGG-3'</td>
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</tr>
<tr>
<td>5'-GGATAAACCCATCCGAGAAGTGTGAGATGCTGACAG-3'</td>
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<tr>
<td>5'-GCTATAGACGCTTGTCAATGTGACTTCACCAGTGG-3'</td>
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</tr>
<tr>
<td>5'-ACCCAGTGGCGCTACATGCTGAGGAAAAACTG-3'</td>
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<tr>
<td>5'-GAAAACCTGAAAGAGGATAACAGAAGGTTAAAAGCTCAGTCACG-3'</td>
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<td>5'-CCTCCAAATATAGGGAACATATGCTTAAAAGGAAAGGATACAGG-3'</td>
<td>12</td>
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**Table 2.1**  Primers for Calpastatin Subcloning and Mutagenesis

Primer pairs used for amplification by PCR of (1) full length human calpastatin or (2) calpastatin domain I adding 5' Ndel and 3' EcoRI restriction sites. Restriction sites are underlined. The sense strand of the mutagenic primer pairs used to delete (3) 4 bp (bp 181-184) in the pET24a multiple cloning site to shift the His-tag in frame and (4) 204 bp of the 3' end of calpastatin domain I to create subdomain A constructs (∗). The sense strand of the mutagenic primer pairs used to change codons (5) C24S TGT→AGT, (6) S142C TCT→TGT, (7) S148C TCG→TGC, (8) S151C TCA→TGT, (9) S235C TCT→TGT, (10) A246C GCT→TGT and (11) S256C TCT→TGT. (12) The sense strand of the mutagenic primer pair used that changed 3 bp (TAT→ATA) to create an Ndel site in calpastatin domain I. Mutated codons are marked blue in primer sequence.
Figure 2.1  Diagram of Subcloning Strategy for Calpastatin Peptide Variants
site (bp 181-184) were deleted to put the His-tag in frame (table 2.1, #3). Four mutations were introduced into calpastatin domain I using the Quik Change II XL Site Directed Mutagenesis Kit and transformed into XL10Gold ultracompetent cells (Stratagene) (table 2.1, #5-8) (figure 2.1 A & B). These mutations included: C24S (TGT→AGT), S142C (TCT→TGT), S148C (TCG→TGC), and S151C (TCA→TGT). The resulting four constructs containing variants of the calpastatin domain I sequence were designated by their new residues S241, C142S241, C148S241, or C151S241.

**Subcloning and Mutagenesis of Calpastatin Domain I Subdomains A and C**

Calpastatin domain I subdomain C sequence (Pro203 to Ser279) was deleted using Quik Change mutagenesis to leave the calpastatin domain I subdomain A sequence encoding Val11 to Pro202 (table 2.1, #4). This deletion was performed in each of the four calpastatin domain I constructs to create four calpastatin domain I subdomain A constructs (figure 2.1 B). Changing three base pairs (TAT→ATA) by site directed mutagenesis created a new internal Ndel site in the calpastatin domain I S241 and C241 sequences (table 2.1, #12) (figure 2.1 C). Digestion with Ndel removed the region encoding Val11 to Leu209. Religation created two calpastatin domain I subdomain C constructs S241 and C241 (encoding Ala210 to Ser279). Using calpastatin domain I subdomain C S241 as template, codons for S237C (TCT→TGT), A246C (GCT→TGT) and S256C (TCT→TGT) were individually mutated by Quik Change mutagenesis (table 2-1, #9-11). The five constructs containing the calpastatin domain I subdomain C sequence are designated by their new residues S241, C237S241, S241C246, and S241C256 or by the WT codon C241.

**Mutagenesis of Calpain 2 Large Subunit and Creation of eGFP Fusion Protein**

Thirteen base pairs of the pET24d multiple cloning site in the rat calpain 2 C105A pET24 plasmid were deleted to allow synthesis of the plasmid encoded carboxy-terminal His-tag (table 2.2, #1). The sequence encoding the tetra-cys motif (ACCPGCCA) was inserted at the 3’ end of
calpain 2 C105A cDNA by Quik Change mutagenesis (table 2.2, #2). The DNA encoding eGFP was digested from the pEGFP vector using HindIII and NotI. The eGFP sequence was ligated into the HindIII and NotI sites between the sequence encoding calpain 2 C105A and the Hexa-His tag of the pET24 plasmid. Insertion of two base pairs (GG) before the eGFP stop codon allowed synthesis of calpain-eGFP including a carboxy-terminal His-tag (table 2.2, #3).

### Table 2-2 Primers for Calpain 2 Mutagenesis

<table>
<thead>
<tr>
<th>Site Directed Mutagenesis</th>
<th>Primer Sequences</th>
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</tr>
<tr>
<td></td>
<td>5'-GCCGCACTCGAGGCctGCTGCCCCGGGTGCTGCGCCCACCACCAC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CGAGCTGTACAAGGTTAAAGCGCCGCAC-3'</td>
</tr>
</tbody>
</table>

(1) The sense strand of the mutagenic primer pair used to delete 13 bp (∆) of 3' sequence between calpain 2 C105A coding sequence and pET24 vector to remove a stop codon and shift the vector encoded His-tag into frame. (2) The sense strand of the mutagenic primer pair used to insert 24 bp (blue) encoding the tetra-cysteine motif between the vector multiple cloning site and His-tag of the construct containing calpain 2 C105A large subunit. (3) The sense strand of the mutagenic primer pair used to insert 2 bp (blue) to shift the eGFP stop codon out of frame and pET24 His-tag in frame in calpain 2 C105A-eGFP construct.

### Expression of Calpain and Calpastatin Constructs

All calpastatin constructs were transformed into *Escherichia coli* strain BL21(DE3) competent cells (Novagen); the calpain 2 constructs were co-transformed with the plasmid encoding truncated rat recombinant small subunit in a modified pET vector designated pACpET (15). Transformed cells were selected on Luria broth (LB)-agar plates (1.6%) containing either kanamycin (10 µg/mL, calpastatin) or kanamycin (10 µg/mL) and ampicillin (50 µg/mL, calpain) at 37°C for 16-18 hours (overnight (O/N)). Isolated colonies were used to inoculate LB with either kanamycin (50 µg/mL, calpastatin) or kanamycin (50 µg/mL) and ampicillin (100 µg/mL, calpain) and incubated shaking at 250 rpm at 37°C O/N. Permanent cell stocks were stored in 50% media and 25% glycerol at -80°C. LB with selective antibiotics was inoculated with mature
culture (8.5 fold dilution) and incubated shaking at 37°C to an optical density of 0.8-1.0 at 600 nm. The transformed cells were induced to express protein or peptide using isopropyl-beta-D-thiogalactopyranoside (IPTG) (0.5 mM) and incubated shaking (200-250 rpm) at room temperature (RT) O/N. The cells were centrifuged (CF) at 4°C 15 minutes in a JLA10.5 rotor at 4,000 rpm, washed in 0.15 M NaCl and CF at 4°C, 15 minutes using a JA25.5 rotor at 5,000 rpm. The supernatant was aspirated and the wet cell pellets were stored at -80°C.

Purification of Recombinant Expressed Calpastatin Peptides

Cell pellets were resuspended in 25 mM 4-morpholinepropanesulfonic acid (MOPS) pH 8.0, 10 mM imidazole, 0.10 M sodium chloride (NaCl), 0.4 mM ethylene glycol bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 0.2 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM β-mercaptoethanol (β-ME) (Ni Binding Buffer (NiBB)) (0.1-0.16 g wet cell weight per mL); subjected to three freeze-thaw cycles (-80°C to RT) and CF at 4°C for 20 minutes in a JA25.5 rotor at 17,000 rpm. The supernatant was passed through Ni-NTA agarose resin (Qiagen) previously packed and equilibrated in NiBB at 4°C with a flow rate of ~8 sec/drop. The resin was washed with NiBB prior to elution of bound peptide with 100 mM imidazole in NiBB, and a second elution with 250 mM imidazole in NiBB (Ni Elution Buffer (NiEB)). The protein concentration was monitored using Bradford reagent (Biorad). The eluted protein was pooled and dialyzed against 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.0, 1 mM tris (carboxymethyl) phosphine (TCEP) and stored at -20°C.

Fluorogenic Modification of Calpastatin Peptides

The calpastatin peptides were labeled according to the protocol in the Thiol-Reactive Probes manual from Molecular Probes/Invitrogen. Tetramethylrhodamine-5-maleimide (TMR), Alexa Fluor 546-5-maleimide, Alexa Fluor 568-5-maleimide, or Alexa Fluor 647-2-maleimide
were resuspended in dimethylsulfoxide (DMSO) to 10.4 mM. Stirring at RT, a 13 fold molar excess of fluorophore was added dropwise to calpastatin peptide (40-80 μM) in 50 mM HEPES pH 7.0, 1 mM TCEP. The reaction was incubated at RT for 1 hour. A 5 molar excess of β-ME to total fluorophore was added to stop the reaction by reacting with unbound fluorophore. Fluorophore labeled peptide was dialyzed extensively against 25 mM MOPS pH 7.5 using Tube-O-Dialyzers (4000 or 8000 MWCO tube-O-dialyzers, Genotech). The stoichiometry of fluorogenic modification was determined using equation (2.1):

\[
\frac{f}{p} = \frac{A_{\lambda}}{\varepsilon(C_p)}
\]  

(2.1)

where \(f/p\) is the ratio of fluorophore per peptide, \(A\) is the fluorophore absorbance at fluorophore’s absorbance wavelength, \(\varepsilon\) is the fluorophore’s extinction coefficient (cm\(^{-1}\) M\(^{-1}\)), and \(C_p\) is the peptide molar concentration. Extinction coefficients used were 95,000 cm\(^{-1}\) M\(^{-1}\) for TMR at 541 nm, 93,000 cm\(^{-1}\) M\(^{-1}\) for Alexa Fluor 546 at 554 nm, 92,000 cm\(^{-1}\) M\(^{-1}\) for Alexa Fluor 568 at 575 nm or 265,000 cm\(^{-1}\) M\(^{-1}\) for Alexa Fluor 647 at 651 nm (Molecular Probes-Stratagene). Peptides labeled with fluorophore were stored at -80°C.

**Purification of Calpain 2 C\(_{105A}\)**

*Escherichia coli* strain BL21(DE3) cells previously induced to co-express the small subunit construct and one of the constructs containing calpain 2 C\(_{105A}\) were resuspended in 50 mM MOPS pH 7.5, 10 mM EGTA, 10 mM EDTA, 10 mM β-ME (0.12-0.24 g wet cell weight per mL). Phenylmethylsulfonyl fluoride (PMSF) was added to the cell suspension (50 μg/mL) and cells were lysed by sonication (Branson Sonifier 450), on ice, for 6-8 minutes (duty cycle 50-60, output 6). The soluble proteins were recovered as supernatant following centrifugation at 4°C for 20 minutes with a JA25.5 rotor at 17,000 rpm. The supernatant was applied to DEAE Sephacel resin previously packed and equilibrated in 50 mM MOPS pH 7.5, 2 mM EGTA, 2 mM EDTA, 5 mM β-ME (DEAE Binding Buffer (DEAE BB)) at 4°C with a flow rate of ~3 sec/drop.
The DEAE resin was washed with DEAE BB followed by 0.15 M NaCl in DEAE BB. The bound calpain was eluted using 50 mM MOPS pH 7.0, 0.5 M NaCl, 2 mM EGTA, 2 mM EDTA, 5 mM β-ME (DEAE Elution Buffer (DEAE EB)). The DEAE eluted protein was pooled and chromatographed on Reactive Red Agarose (RRA) resin previously packed and equilibrated in DEAE EB at 4°C with a flow rate of ~3 sec/drop. The RRA resin was washed with DEAE EB while maintaining the same flow rate. The bound protein was eluted using cold deionized distilled water (8 sec/drop flow rate). The calpain was eluted from RRA into tubes containing concentrated NiBB (50 mM imidazole) for C105A H6 and C105A tetra-cys H6 or concentrated DEAE BB for C105A-eGFP. The eluted calpain diluted the NiBB (5 mM imidazole) and DEAE BB to concentrations specified above. Calpain C105A H6 and C105A tetra-cys H6 in NiBB were bound to Ni-NTA agarose resin previously packed and equilibrated in NiBB at 4°C with a flow rate of ~8 sec/drop. The bound C105A H6 and C105A tetra-cys H6 were washed with NiBB and eluted with NiEB. The C105A-eGFP in DEAE BB was bound to DEAE Sephacel resin packed and equilibrated in DEAE BB at 4°C with a flow rate of ~3 sec/drop. The bound protein was washed with DEAE BB then eluted with DEAE EB. The protein concentration was monitored using Bradford reagent (Biorad). Purified C105A H6 and partially purified C105A-eGFP were dialyzed against 50 mM MOPS pH 7.5, 1 mM EGTA, 1 mM EDTA, 0.5 mM dithiothreitol (DTT). Purified C105A tetra-cys H6 was dialyzed against 50 mM MOPS pH 7.5, 1 mM TCEP. Aliquots of purified calpains were flash frozen using liquid nitrogen, and stored at -80°C.

**Preparation of Calpain 2-eGFP Crude Lysates**

A pellet of BL21(DE3) cells that were induced to co-express the small subunit construct and the construct containing calpain 2 C105A-eGFP was resuspended, sonicated, and centrifuged as explained for calpain purification above. Total protein was quantified using Bradford reagent. The concentration of C105A-eGFP was determined by absorbance at 508 nm using the eGFP
extinction coefficient of 55,900 cm$^{-1}$ M$^{-1}$. Aliquots of crude lysates were flash frozen using liquid nitrogen and stored at -80°C.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

SDS-PAGE was executed according to Schagger and von Jagow (48). Following electrophoresis gels were soaked in fixative reagent (50% methanol, 10% acetic acid); stain (0.1% Coomassie, 0.05% amido black, 40% methanol, 10% acetic acid); destain (25% methanol, 10% acetic acid) until the background was clear and stored in distilled deionized water. Images of gels were recorded using the ChemiImager 4400 Low Light Imaging System (Alpha Innotech Corporation).

**Fluorogenic Modification of Calpain 2 C105A Tetra-Cys**

A working solution of 1 mM FlAsH (Lumio Green, Invitrogen), 10 mM EDT, 92% DMSO was prepared immediately prior to use. FlAsH working solution was added to purified calpain 2 C105A tetra-cys H6 in 50 mM MOPS pH 7.5, 1 mM TCEP at a ratio of 2 moles of FlAsH per mole of calpain and incubated at 4°C O/N (1% DMSO). The FlAsH labeled calpain was dialyzed extensively against 50 mM MOPS pH 7.5, 1 mM EGTA, 1 mM EDTA, 0.5 mM DTT. The stoichiometry of fluorogenic modification was determined using equation (2.1) and the FlAsH extinction coefficient of 41,000 cm$^{-1}$ M$^{-1}$ at 508 nm. The calpain concentration was estimated using the absorbance at 280 nm and the extinction coefficient 117,160 cm$^{-1}$ M$^{-1}$. Aliquots of C105A-FlAsH were flash frozen using liquid nitrogen, and stored at -80°C.

**Measuring FRET in Response to Increasing Acceptor-Calpastatin**

Initial donor emission C105A-FlAsH (2 μM) or C105A-eGFP (1 μM) was established in the absence of acceptor-calpastatin by exciting at 460 (C105A-FlAsH) or 440 nm (C105A-eGFP) nm
while scanning for emission between 480 (or 495) to 650 nm using a Perkin-Elmer luminescence spectrometer LS 50 B (4.0-5.0 nm slits). Emission scans were performed following addition of 1) acceptor labeled calpastatin domain I (1 µM), 2) calcium (8-10 mM), 3) additional aliquots of acceptor-calpastatin domain I (up to 6 µM (C10/A-FlAsH) or 12 µM (C10sA-eGFP)), and 4) EGTA (18-20 mM) to show reversal of binding. Five scans were performed after each addition. Data from the five scans were averaged (using MatLab) and the areas under the donor (510-545 nm, FlAsH; 480-540 nm, eGFP) and acceptor (560-620 nm, FlAsH-TMR; 550-620 nm, eGFP-AF546) emission peaks was calculated according to equation (2.2):

\[ A = \sum y \]  

where A is the area and y is the fluorescence intensity between the designated wavelengths (at 0.5 nm intervals).

**Measuring FRET in Response to Increasing Calcium**

Initial donor emission C10sA-FlAsH (0.75 µM) or C10sA-eGFP (1 µM) in 50 mM MOPS pH 7.5, 0.25 M NaCl, 1 mM TCEP was established in the absence of acceptor-calpastatin by exciting at 460 nm while scanning for emission between 480 (or 495) to 650 nm (4.0-5.0 nm slits). Emission scans were performed following addition of 1) acceptor-calpastatin domain I (1.5-2 µM), 2) calcium (up to 2 mM), and 3) EGTA (12 mM) to show reversal of binding. Five scans were performed after each addition. Data from the five scans were averaged (using MatLab) and the ratio of RFU at the acceptor emission peak (TMR or Alexa Fluor 546, 570 nm) over RFU of the donor emission peak (eGFP, 508 nm; FlAsH 528 nm) (EmA/EmD) was calculated. The free calcium concentrations for each data collection point were calculated via WEBMAXC Standard available through Maxchelator. The EmA/EmD ratios were plotted vs. the
free calcium concentration. The percent increase in the calcium dependent signal was calculated using equation (2.3),

\[
\text{% increase} = \left( \frac{R_f R_i}{R_f} \right) (100)
\]  

(2.3)

where \( R_i \) is the \( E_{mA}/E_{mD} \) ratio at 0 \( \mu \)M free calcium and \( R_f \) is the average \( E_{mA}/E_{mD} \) ratio at 0.8-2 mM free calcium (saturation). The increase in ratio for the amino-terminally labeled acceptor-calpastatin peptides or nonspecifically labeled acceptor-peptides were averaged for each donor-calpain and expressed as an average percent ± the average difference.

**Measuring FRET in Response to Binding of Fluorogenic Calpastatin Subdomain Peptides to Calpain**

Initial donor emission of calpastatin domain I subdomain C-AF568 (0.5 \( \mu \)M) in 50 mM MOPS pH 7.5, 0.25 M NaCl, 1 mM TCEP and calpain 2 C\textsubscript{105}A (1 \( \mu \)M) was established by exciting at 515 nm while scanning for emission between 570 to 720 nm. Emission scans were performed following addition of 1) calpastatin domain I subdomain A-AF647 (2 \( \mu \)M), 2) calcium (11 mM), and 3) EGTA (22 mM) to show reversal of binding. Five scans were performed after each addition using 2.5 nm excitation slits and 5.0 nm emission slits. The five scans were averaged (using MatLab) and the ratio of RFU at the acceptor emission peak (AF647 665 nm) over RFU of the donor emission peak (AF568 594 nm) \( (E_{mA}/E_{mD}) \) was calculated. The percent increase in the calcium dependent signal was calculated according to equation (2.3).

**Measuring FRET in Response to Unmodified Calpastatin, Calpains, or Calpain Substrates**

Initial donor emission C\textsubscript{105}A-eGFP (1 \( \mu \)M) in 50 mM MOPS pH 7.5, 0.25 M NaCl, 1 mM TCEP was established in the absence of acceptor-calpastatin by exciting at 460 nm while
scanning for emission between 480 to 650 nm using 4.8 (β integrin & α-casein) or 5.0 (calpastatin) nm slits. Emission scans were performed following addition of 1) calpastatin domain I C$_{148}$S$_{241}$-AF546 (1 µM) with calpastatin domain I S241 (0-2 µM), β integrin cytoplasmic tail (0-20 µM) or α-casein (0-20 µM), 2) calcium (10 mM), and 3) EGTA (20 mM) to show reversal of binding. In a similar series of assays, the initial donor emission C$_{105}$A-eGFP (0.5-1 µM) with calpain 1 (0-1 µM) or calpain 2 C$_{105}$A H6 (0-2 µM) in 50 mM MOPS pH 7.5, 0.25 M NaCl, 1 mM TCEP was established in the absence of acceptor-calpastatin by exciting at 460 nm while scanning for emission between 480 to 650 nm using 5.0 (calpain 2) or 8.0 (calpain 1) nm slits. Emission scans were performed following addition of 1) calpastatin domain I C$_{148}$S$_{241}$-AF546 (0.5-1 µM), 2) calcium (10 mM), and 3) EGTA (20 mM) to show reversal of binding. Five scans were performed after each addition. The data from the five scans were analyzed as previously described on pg 21.
Constructing Fluorogenic Proteins

Subcloning and Mutagenesis of Calpastatin Peptides  In an initial strategy to develop a FRET sensor for detecting active calpain, calpastatin was modified to allow specific labeling with acceptor fluorophores. Calpastatin domain I was chosen for use based on the premise that one inhibitory domain binds and inhibits one molecule of calpain. Specifically domain I was chosen due to its relative effectiveness inhibiting calpains 1 and 2 (I>IV>III>II) (27). The chemistry of maleimide coupling to thiol groups allows specific modification of cysteine residues when using maleimide conjugated fluorophores. To employ these fluorophores and specifically modify the amino-terminus of calpastatin domain I, constructs expressing calpastatin domain I with a single, distinctly located cysteine residue near the amino-terminus were engineered. Calpastatin domain I, Val114 to Ser270 (numbering as in whole calpastatin), was subcloned from full length human calpastatin into the pET24a expression vector. Naturally occurring domain I contains one cysteine residue at position 241 (C241) (figure 3.1). To allow specific labeling near the amino-terminus of calpastatin domain I, cysteine 241 was first mutated to encode a serine. Using this cysteine free construct, three amino-terminal serine residues at positions 142, 148, and 151 were individually changed to cysteines. The four different calpastatin domain I constructs generated include three constructs each encoding one, distinct amino-terminally located cysteine residue S142C C241S, S148C C241S, or S151C C241S and one control construct encoding no cysteine residues C241S. These constructs and the peptides expressed from them are designated by the new residues S241, C142S241, C148S241, or C151S241.
Human calpastatin domain 1 amino acid sequence as numbered from whole calpastatin. The amino acid residues altered by site directed mutagenesis are shown in blue and numbered. Calpastatin domain I subdomain A peptides consisted of residues 114 to 202 (red underlined) and calpastatin domain I subdomain C peptides consisted of residues 210 to 270 (green underlined). The key inhibitory sequence of the subdomain B peptide mimics is overlined (grey). Calpastatin domain I and subdomain A peptides are expressed with a Met before Vn4 while calpastatin domain I subdomain C peptides are expressed with a Met before A210.

In an alternative strategy to develop a FRET sensor for detecting active unmodified calpain, constructs to express calpastatin domain I subdomain A and subdomain C peptides were created and modified to allow specific labeling with maleimide fluorophores. To develop the constructs expressing calpastatin domain I subdomain A, the four calpastatin domain I constructs S241, C142S241, C148S241, and C151S241 were used as templates for mutagenesis to delete the 3’ carboxy-terminal sequence encoding Pro203 to Ser270. This resulted in three calpastatin domain I subdomain A constructs each encoding one distinctly located amino-terminal cysteine residue and one construct encoding no cysteine residues (figure 3.1). To generate constructs expressing calpastatin domain I subdomain C, mutagenesis introduced a new internal Ndel site in the constructs containing calpastatin domain I C241 or S241. Subsequent digestion with Ndel and religation allowed removal of the sequence encoding Val114 to Leu209. This produced constructs expressing calpastatin domain I subdomain C encoding the naturally occurring (WT) cysteine residue at position 241 (C241) or encoding no cysteine residues (S241). Serine 237, alanine 246, or serine 256 were mutated to cysteines in calpastatin domain I subdomain C S241. Thus there are four calpastatin domain I subdomain C constructs each encoding one distinctly located cysteine.
residue; $S_{237}C_{241}S$, $C_{241}S_{246}C$, and $C_{241}S_{256}C$ (figure 3.1). These constructs are designated by either their new codons $C_{237}S_{241}$, $S_{241}C_{246}$, and $S_{241}C_{256}$ or by the WT codon $C_{241}$.

**Purification and Fluorogenic Modification of Calpastatin Peptides**

The four calpastatin domain I constructs, four calpastatin domain I subdomain A constructs and four calpastatin domain I subdomain C constructs were each expressed in the BL21(DE3) *Escherichia coli* (*E. coli*) expression strain. After induction of expression by IPTG, cells resuspended in buffer (see methods) were subjected to three cycles of being frozen at -80°C and then thawed at room temperature. This exploited the expressed peptides’ small size to be released from the cells with minimal cell lysis. Comparing the supernatant released from these cells after being repeatedly frozen and thawed (S) to the protein contents remaining in the cells (L) demonstrated release of small peptides in the supernatant (figure 3.2 A). The peptides were purified utilizing the expressed peptides’ carboxy-terminal hexa-histidine affinity tag to bind Ni agarose under native conditions. This produced relatively pure peptides with the theoretical molecular weight of approximately 18.7 kDa for domain I (figure 3.2), 11.7 kDa for subdomain A, or 8.4 kDa for subdomain C (figure 3.2 B). The purified calpastatin peptides however did not migrate true to their size but appeared larger (~27 kDa for domain I, ~17 kDa for subdomain A, or ~12 kDa for subdomain C) than the theoretical molecular weight on the SDS polyacrylamide gels. This phenomenon is characteristic of intrinsically unstructured proteins and intact calpastatin (54) further supporting successful purification of the engineered calpastatin peptides.

Using the specificity of maleimide’s reactivity with thiol groups and the lone cysteines specifically engineered in the peptides the four purified domain I peptides were labeled with tetramethylrhodamine (TMR)-5-maleimide or Alexa Fluor 546 (AF546)-5-maleimide. The four purified subdomain A peptides were labeled with Alexa Fluor 647 (AF647)-2-maleimide or Alexa Fluor 546-5-maleimide and the four purified subdomain C peptides were labeled with
Figure 3.2 SDS-Polyacrylamide Gel Electrophoresis of Recombinant Calpastatin Peptides

A) *E. coli* expressing recombinant human calpastatin domain I S_{241} suspended in buffer were subjected to three freeze/thaw cycles. The supernatant was separated from the intact cells and loaded on Ni resin for purification under native conditions. To monitor progression of peptide isolation, aliquots at each stage of the purification procedure were treated with SDS sample buffer and analyzed by electrophoresis. Aliquots include lysate from cells post freeze/thaw (L), supernatant post freeze/thaw (S), Ni unbound (U), Ni elution (E) and Ni elution shoulder (Sh). Migration of molecular weight markers (Pharmacia) is indicated in kDa. The purification progress is representative for all calpastatin peptides purified. Bands corresponding to the calpastatin domain I peptide is marked by an arrow. 

B) Recombinant human calpastatin domain I subdomain A C_{131} (A) and subdomain C S_{241}C_{255} (C) peptides (5 µg) were purified as described for domain I. Size and purity of purified subdomains shown are representative for the eight purified subdomain A and C peptides. Bands corresponding to calpastatin subdomain A and C peptides are marked with arrows. Migration of markers is indicated in kDa and based on a combination of low molecular weight markers (Pharmacia) and Protein Markers (USB). Gels pictured are 9% acrylamide and stained with Coomassie blue-amido black.
Alexa Fluor 568 (AF568)-5-maleimide or Alexa Fluor 546-5-maleimide (see methods). As shown in table 3-1 this created eight acceptor fluorophore labeled domain I peptides, eight acceptor labeled subdomain A peptides, four donor labeled and four acceptor labeled subdomain C peptides.

The stoichiometry of the fluorophore labeled calpastatin peptides was determined to assess the extent of fluorophore modification. Stoichiometry was determined by comparing the estimated peptide concentration after labeling to the estimated concentration of the fluorophore. The fluorophores were quantified using published extinction coefficients. The calpastatin concentration post-labeling was estimated based on the concentration prior to labeling. Spectral overlap of the AF546 and TMR absorbance with the absorbance wavelength for the Bradford reagent prevented accurate quantification of acceptor-calpastatin. Quantification of the fluorophores using extinction coefficients is more accurate than quantification of calpastatin by Bradford and enhanced BCA assays (11). The stoichiometry of fluorophore per estimated mole of cysteine containing peptide indicated a 2:1 to 4:1 ratio even though each peptide contained only one cysteine residue for modification. A ratio greater than 1:1 indicates that either the calpastatin peptide concentration is inaccurate or there is an occurrence of nonspecific coupling of fluorophore to residues other than cysteines. The combination of an accurate fluorophore quantity and potential underestimation of the calpastatin peptides could produce an inaccurate fluorophore to peptide ratio. However, nonspecific labeling by maleimide conjugated fluorophores has been reported (4, 51). To test for nonspecific modification calpastatin domain I S241 and subdomain A which both lack cysteine residues, were treated with TMR-5-maleimide and/or AF546-5-maleimide. Modification of both peptides by the maleimide fluorophores was observed. Specifically calpastatin domain I S241 was modified by TMR with a stoichiometry of approximately 0.5:1 (n=1). These observations confirm the occurrence of some nonspecific
Table 3-1  *Compilation of Fluorogenic Proteins*
Full list of fluorogenic proteins developed and tested for FRET biosensors to detect active calpain. The calpastatin peptides are designated by their new residues. The control peptides are marked (*).

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<thead>
<tr>
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<th>Acceptor–Fluorophores</th>
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Table 3-1  *Compilation of Fluorogenic Proteins*
Full list of fluorogenic proteins developed and tested for FRET biosensors to detect active calpain. The calpastatin peptides are designated by their new residues. The control peptides are marked (*).
labeling. The calpastatin domain I S241-AF546, domain I S241-TMR and subdomain A-AF546 peptides served as controls for the nonspecific coupling of fluorophore to residues other than site specific cysteines in FRET assays.

**Generating Donor Modified Calpains** Specific labeling of the carboxy-terminus of the large subunit of calpain 2 required a different strategy. FlAsH (Lumio Green-Invitrogen), a fluorescein modified with two arsenic groups, specifically binds a tetra-Cys motif (CCXXCC) (65). This rare motif is not found in native calpain 2. The sequence encoding ACCPGCCA was inserted by site directed mutagenesis 5' to the pET24 vector hexa-histidine tag at the carboxy-terminus of domain IV of the inactive calpain 2 mutant C_{105}A and designated C_{105}A tetra-cys (C_{105}A-4C). Alternatively, the sequence encoding eGFP was fused to the carboxy-terminus of calpain 2 C_{105}A domain IV and designated C_{105}A-eGFP. Each of these constructs was co-expressed with a plasmid encoding the recombinant rat calpain small subunit in *E. coli* and purified as intact heterodimeric proteins (methods). The C_{105}A-eGFP subunits although only partially purified are evident by SDS-PAGE gel (figure 3.3). These consist of the 21 kDa small subunit and 109 kDa fusion protein of 80 kDa calpain 2 C_{105}A large subunit and 29 kDa eGFP. The eGFP is separated from the calpain large subunit by a 20 residue linker region while a 7 residue linker separates eGFP from the pET24 vector encoded hexa-histidine tag. The 80 kDa large and 21 kDa small subunits of the purified C_{105}A-4C can be distinguished on an SDS-PAGE gel (figure 3.3). FlAsH was bound to the tetra-cysteine motif of purified C_{105}A-4C as described in methods and redesignated C_{105}A-FlAsH. Fluorophore and calpain concentrations post-labeling were determined utilizing extinction coefficients for FlAsH at 508 nm and calpain at 280 nm absorbance. The stoichiometry of labeling was determined to range from 1:2 to 1:1 FlAsH per calpain based on these concentrations (n=3). Thus inactive calpain 2 labeled at the large subunit carboxy-terminus with two different donor fluorophores, FlAsH and eGFP, was generated.
Figure 3.3  
**SDS-Polyacrylamide Gel Electrophoresis of Recombinant Calpains**
Purified recombinant rat calpain 2 C105A (1), C105A tetra-cysteine (2) and partially purified C105A-eGFP (3) (4 μg each). Bands corresponding to the calpain large (~80 or 109 kDa) and small (~21 kDa) subunits are marked with arrows. Migration of molecular weight markers (Pharmacia) is indicated in kDa. Gel pictured is 9% acrylamide and stained with Coomassie blue-amido black.

**Demonstrating Calcium Dependent Detection of Calpain using FRET**

*A Two Component Strategy for Detecting Active Calpain*  
The two different designs of a sensor to detect active calpain both exploit the calcium dependent, specific binding of calpastatin to the conventional calpains. The two component strategy places the acceptor fluorophores AF546 or TMR at the amino-terminus of calpastatin domain I and the donor fluorophores FlAsH or eGFP at the carboxy-terminus of calpain 2 C105A domain IV. In the presence of calcium the acceptor-calpastatin is expected to bind donor-calpain bringing the fluorophore pair within approximately a 31 Å distance. This distance should allow FRET between the fluorophore pair. Two different approaches were used in binding assays to test for a FRET generating pair of fluorogenic proteins. The initial approach was to add aliquots of acceptor-calpastatin domain I to 1 or 2 μM donor-calpain in the presence of calcium. Using a donor specific excitation
wavelength (460 nm), changes in the donor and acceptor emission spectra (480 or 495 to 650 nm) were monitored as the acceptor-calpastatin concentration increased. Results representative for the twelve pairs are shown using an acceptor-calpastatin modified at an amino-terminal cysteine as acceptor to a donor-calpain (figure 3.4). The donor emission peak in these scans decreases and the acceptor emission peak increases as the acceptor-calpastatin domain I concentration increases. Both donor quenching and increasing acceptor emission are indicative of successful FRET. The most sensitive measure of FRET is expressed as a ratio of acceptor emission over donor emission \( \frac{E_{mA}}{E_{mD}} \). As expected the \( \frac{E_{mA}}{E_{mD}} \) increased in response to the increasing acceptor-calpastatin but did not demonstrate saturation of binding to the limited amount of donor-calpain (figure 3.5). Direct excitation of the acceptor fluorophore or dilution of the donor fluorophore both may contribute to preventing saturation of the ratio.

There are two components that contribute to the increase of the ratio; quenching of the donor emission and an increase in acceptor emission. Each addition of acceptor-calpastatin domain I dilutes the donor-calpain throughout the assay. This dilution may contribute to the apparent donor quenching. To determine the effect of dilution on the donor emission peak, the change in the area of the donor emission peak for control assays using unmodified calpastatin domain I \( S_{241} \) was compared to that of FRET assays using acceptor-calpastatin. The area of the FlAsH donor emission peak decreased 3.95±0.05 (n=2) in the presence of calpastatin domain I \( C_{151}S_{241}-TMR \) but only 1.01 (n=1) in the presence of unmodified calpastatin domain I \( S_{241} \) (0-10 \( \mu \)M) (figure 3.6 A). The area of the eGFP donor emission peak decreased 4.86 (n=1) in the presence of calpastatin domain I \( C_{142}S_{241}-AF546 \) but only 1.86±0.38 (n=2) in the presence of unmodified calpastatin domain I \( S_{241} \) (0-10 \( \mu \)M) (figure 3.6 B). These results demonstrate the area of the donor emission peaks decreased substantially in the presence of acceptor-calpastatin domain I. The area of the donor also decreased in the presence of unmodified domain I although
Figure 3.4  **Representative Emission Scans of Donor-Calpains in the Presence of Increasing Concentrations of Acceptor-Calpastatin**

Initial donor emission for A) C105A-FlAsH (2 μM) or B) C105A-eGFP (1 μM) was established in the absence of acceptor-calpastatin by exciting at 460 nm (A) or 440 nm (B) while scanning for emission between 495 (A) or 480 (B) to 650 nm using 5.0 nm slits. Emission scans were performed following addition of 1) calpastatin domain I C151S241-TMR (1 μM), 2) calcium (14 mM), 3) additional aliquots of calpastatin domain I C151S241-TMR (2-10 μM), and 4) EGTA (21 mM) to show reversal of binding. Insets designate individual scans for each step in the binding assays. Results in each panel are representative for the six different acceptor modified calpastatin peptides studied.
Figure 3.5  FRET Response Relative to the Concentration of Acceptor-Calpastatin Peptide
The ratio of acceptor emission over donor emission was used to assess FRET relative to acceptor-calpastatin concentration for a series of binding assays. Using data from assays similar to those in figure 3.4, the E_{mA}/E_{mD} ratio was determined for A) C_{106A-FlAsH} (575 nm/528 nm) or B) C_{108A-eGFP} (575 nm/508 nm) as donor to one of the six acceptor-calpastatin domain I peptides in the presence of calcium. Insets designate symbols for the different acceptor-calpastatin domain I peptides. Results shown are representative of two assays unless otherwise indicated (1) in insets.
Figure 3.6  Controls for Dilution and Direct Excitation Effects
Assessing the contribution of dilution of the donor emission intensity (left) and the contribution of acceptor direct excitation on acceptor emission intensity (right) due to increasing calpastatin domain I. The area of the donor and acceptor peaks is calculated as the sum of intensity. A) C105A-FlAsH (2 μM) (510-545 nm) serves as donor to calpastatin domain I C151S241-TMR (n=2) (circles) or calpastatin domain I S241 (squares) (560-620 nm). B) C105A-eGFP (1 μM) (480-540 nm) serves as donor to calpastatin domain I C142S241-AF546 (circles) or calpastatin domain I S241 (squares) (n=2, donor emission control) (550-620 nm). Based on a single assay unless otherwise noted. ‡ indicates individual control measurements that have been duplicated.
not to the extent as for acceptor-calpastatin. This suggests that FRET is the major factor of the observed donor quenching.

Direct excitation of the acceptor fluorophore likely contributed to the increasing acceptor emission peak due to the fact that the ratio does not reach saturation despite the fixed amount of donor-calpain. As the donor emission reaches maximal quenching, a continual increase in acceptor emission due to direct excitation would increase the ratio. To assess the contribution of direct excitation to the signal, the acceptor-calpastatin emission response to the donor excitation wavelength was measured in the absence of donor-calpain. The area of the acceptor emission peak increases as the acceptor-calpastatin concentration increases indicating direct excitation of the acceptor fluorophore in the absence of the donor fluorophore. The calpastatin domain I C_{151}S_{241}-TMR emission peak area increased 2.57±0.38 (n=2) in the presence of C_{105}A-FLAsH but also increased 2.23 (n=1) in the absence of donor calpain (0-10 μM) (figure 3.6 A). The calpastatin domain I C_{142}S_{241}-AF546 emission peak area increased 4.19 (n=1) in the presence of C_{105}A-eGFP but also increased 4.28 (n=1) in the absence of donor calpain (0-10 μM) (figure 3.6 B). The direct excitation acceptor emission peak is lower in the absence of donor calpain. This difference identifies some energy transfer taking place between the donor and acceptor fluorophores. However the continual increase in the acceptor emission due to acceptor direct excitation does not allow saturation of the ratio.

**Calcium Dependence of the Two Component Strategy** A second approach to test for FRET between donor-calpain and acceptor-calpastatin was to add increasing amounts of calcium to fixed concentrations of calpastatin and calpain. As shown in figure 3.4, there was a calcium dependent increase in the emission ratio at the lowest acceptor-calpastatin concentration (1 μM). Based on these findings, calpastatin and calpain were used for subsequent assays at a 2:1 molar ratio to allow direct measurement of binding between the fluorogenic pair of proteins in response
to calcium. Using an excitation wavelength specific to the donor fluorophore (460 nm), emission spectra (480/495 to 650 nm) were collected to monitor the changes in the donor and acceptor peaks in response to the increasing calcium concentration. These spectra demonstrated a dose responsive quenching of the donor emission (figure 3.7). Expressing the donor emission quenching and slight acceptor emission increase as a ratio of $E_{M\delta}/E_{M\theta}$ revealed a cooperative increase. Furthermore, the ratio leveled off as expected demonstrating saturation of ligand binding in this assay format. This calcium dependent increase in the FRET ratio observed from zero free calcium to saturation (0.8-2 mM) was expressed as a percent increase of the ratio. Pooling data and averaging the percent increase for the six different acceptor-calpastatin domain I peptides, there was a 332.56%±48.64 (n=6) increase for $C_{105\text{A}}$-FlAsH and 95.87%±10.02 (n=6) increase for $C_{105\text{A}}$-eGFP as donors. However similar data analysis using the two acceptor-calpastatin control peptides as acceptors only demonstrated a 18.18% (n=1) increase for $C_{105\text{A}}$-FlAsH and 15.09%±1.24 (n=2) increase for $C_{105\text{A}}$-eGFP (figure 3.8). Thus specific placement of the acceptor fluorophores near the amino-terminus of the calpastatin domain I peptides resulted in approximately a 6 fold ($C_{105\text{A}}$-eGFP) or 18 fold ($C_{105\text{A}}$-FlAsH) increase in signal. The ratio increase in response to calcium and decrease in response to EGTA demonstrate the calcium dependence of the FRET signal. These observations validate the signal is a detection of the binding of acceptor-calpastatin to the active conformation of donor-calpain in response to calcium.

**A Strategy for Detecting Active Endogenous Calpain** The two component FRET binding pair requires calpain with a donor fluorophore at the carboxy-terminus of the catalytic subunit. Utilizing this design in situ would require over-expression of $C_{105\text{A}}$-eGFP or $C_{105\text{A}}$-4C followed by in situ labeling using FlAsH. A method for detecting activation of endogenous calpain would detect physiologically relevant calpain in situ. A three component binding system exploiting calpastatin’s calcium dependent interaction with the conventional calpains was designed. This
To assess the changes in donor and acceptor emission in response to the calcium concentration, binding assays were performed using A) C105A-FlAsH (2 μM) or B) C105A-eGFP (1 μM) and calpastatin domain I C148S241-TMR (2 μM) with increasing calcium followed by EGTA addition to reverse binding. Left panels show emission scans (5.0 nm slits) at the free calcium concentrations indicated by the inset. The right panels show the $E_{\text{m}}/E_{\text{D}}$ ratio plotted relative to increasing calcium concentration (circles) and after EGTA addition (squares). Assays shown are representative for the six acceptor-calpastatin peptides studied.
Figure 3.8  Comparison of Binding Assays with Various Donor-Calpain and Acceptor-Calpastatin Pairs

The ratio of acceptor emission over donor emission was used to assess FRET relative to calcium concentration for a series of binding assays. Using data from assays similar to those in figure 3.7, the percent change in ratio was determined from the basal ratio at 0 µM free calcium to the average ratio at saturation (0.8-2 mM). C105A-FLAsH (A) or C105A-eGFP (B) served as donor to each of the acceptor-calpastatin domain I peptides: S241 (light blue), C142S241 (blue), C148S241 (coral), and C151S241 (yellow). Results shown are representative of two assays unless otherwise indicated (‡=1).
design utilizes fluorogenically modified calpastatin domain I subdomain A and subdomain C peptides to bind unmodified calpain 2 C105A. In the presence of calcium AF647-calpastatin subdomain A (acceptor) and AF568-calpastatin subdomain C (donor) are predicted to bind calpain bringing the fluorophore pair within approximately a 51 Å distance. This distance, although greater than the distance between the bound catalytic subunit carboxy-terminus and calpastatin domain I amino-terminus, is within the manufacturer determined 82 Å R0 for AF568 as donor to AF647 (Invitrogen-Molecular Probes). To screen for a combination of fluorogenic calpastatin domain I subdomain A and subdomain C peptides that generate FRET, twelve different pairs of donor-calpastatin subdomain C and acceptor-calpastatin subdomain A peptides were tested in binding assays. Emission spectra (570 to 720 nm) in response to excitation of the donor AF568 fluorophore (515 nm) did not indicate a calcium dependent quenching of the donor AF568 peak or an increase in the acceptor AF647 peak (figure 3.9 A). Thus there was not a detectable transfer of energy between the bound, fluorophore modified subdomains.

The two component strategy utilizing a donor-calpain and acceptor-calpastatin was employed in order to verify that each different subdomain peptide is binding calpain. In a series of assays C105A-eGFP served as donor to all eight AF546 modified calpastatin subdomain A and C peptides. The subdomain A peptides with an amino-terminal AF546 produced an average calcium dependent increase in FRET signal of 41.97%±6.57 (n=3) while the control peptide (subdomain A-AF456) only produced a FRET signal increase of 3.58% (n=1) (figure 3.9 B). This trend was similar to that observed for whole domain I and confirmed the ability of fluorophore modified calpastatin subdomain A peptides to bind calpain. The AF546-subdomain C peptides however showed only a 5.66%±6.31 (n=4) calcium dependent increase in FRET ratio. Thus there was minimal detection of energy transfer between the bound C105A-eGFP and calpastatin domain I subdomain C-AF546 peptides.
Figure 3.9  Preliminary Tests Using Fluorogenic Calpastatin Subdomain Peptides

A) Initial donor emission of calpastatin domain I subdomain C $S_{245}C_{246}$-AF568 (---) was established in the absence of acceptor by exciting at 515 nm while scanning for emission between 570-720 nm using 2.5 nm excitation slits and 5.0 nm emission slits. Emission scans were performed following addition of 1) calpastatin domain I subdomain A $C_{146}$-AF647 (2 μM) (— —), 2) calcium (11 mM) (---), and 3) EGTA (22 mM) (— -) to show reversal of binding. Assay shown is representative for the twelve subdomain C-AF568, subdomain A-AF647 peptide combinations studied. B) To assess binding of acceptor-calpastatin subdomain peptides, changes in the FRET ratio were calculated for a series of binding assays using calpastatin subdomain A-AF546 or subdomain C-AF546 peptides to bind $C_{105}$-eGFP (1 μM). The percent increase in ratio ($E_{m}/E_{0}$) was determined in response to calcium (11 mM) for the eight acceptor-calpastatin domain I subdomain peptides (2 μM). Inset designates the different AF546-calpastatin domain I subdomain peptides, subdomain A (SdA) or subdomain C (SdC). Results shown are from single assays.
In Vitro Applications for a Calpastatin Based FRET Biosensor to Detect Active Calpain

The studies using purified components established the ability to detect the energy transfer upon binding of acceptor-calpastatin to donor-calpain in response to calcium. To begin assessing if this design has the potential to work in the complex cellular environment, a crude BL21(DE3) cell lysate containing C105A-eGFP was used in place of the partially purified C105A-eGFP. Addition of one of the eight different acceptor modified calpastatin domain I peptides in a series of assays using E. coli cell lysate containing C105A-eGFP (0.5 μM) followed by addition of calcium resulted in quenching of the donor peak and an increase in the acceptor peak during emission scans (figure 3.10 A). The percent increase in the EmA/EmD ratio upon calcium addition was calculated for all eight pairs (figure 3.10 B). An average increase in EmA/EmD of 41.24%±5.88 (n=6) was observed for the amino-terminal acceptor-calpastatin domain I peptides relative to a -0.46%±4.54 (n=2) change observed in the presence of nonspecifically modified AF546-calpastatin domain I peptides. These results demonstrated energy transfer can be detected for the binding of acceptor-calpastatin domain I to C105A-eGFP in a crude cell lysate.

In eukaryotic cells, endogenous calpastatin and calpain will both be present and expected to bind the C105A-eGFP or acceptor-calpastatin domain I. In order to gain insight into the impact these proteins may pose to the FRET signal when applied in cells, the ability of calpastatin, calpain 1, or calpain 2 to compete for the binding of calpastatin domain I C148S241-AF546 to C105A-eGFP was determined. The calcium dependent increase in EmA/EmD due to the binding of calpastatin domain I C148S241-AF546 to C105A-eGFP was determined in the presence of unlabeled calpastatin domain I S241, WT calpain 1 or calpain 2 C105A. As expected calpastatin and calpain 2 demonstrated a calcium dependent, dose responsive reduction in the FRET ratio (figure 3.11 A). Importantly though, calpain 1 also displayed a dose dependent reduction in the ratio in
Figure 3.10  Detecting Calpain-eGFP in a Crude Cell Lysate

A) Initial donor emission of a crude lysate from E. coli expressing C105A-eGFP (0.5 μM eGFP) was established in the absence of acceptor-calpastatin by exciting at 460 nm while scanning for emission between 480-650 nm (---) (5.0 nm slits). Emission scans were performed following addition of 1) calpastatin domain I C151S241-TMR (2 μM) (---), 2) calcium (14 mM) (— —), and 3) EGTA (24 mM) (---) to show reversal of binding. Assay shown is representative for the six acceptor-calpastatin peptides studied.

B) Changes in the FRET ratio were calculated for a series of binding assays using crude lysate from E. coli expressing C105A-eGFP (0.5 μM eGFP) as donor to each acceptor-calpastatin domain I peptide (2 μM): S241 (light blue), C142S241 (blue), C148S241 (coral), or C151S241 (yellow). The percent increase in ratio (Emₐ/Em₀) was determined in response to calcium (14 mM). Results shown are representative of single assays for each pair studied.
Figure 3.11  

Unmodified Calpastatin or Calpain Competes with Calpastatin Domain 1 

C148S241-AF546 Binding to C105A-eGFP

To assess the ability of endogenous calpastatin or calpain to compete for the binding of C105A-eGFP to calpastatin domain I C148S241-AF546, unlabeled calpastatin, calpain 1, or calpain 2 were used as competitors.  

A) The change in $E_{m1}$/$E_{m0}$ ratio upon addition of calcium (10 mM) was determined for a series of binding assays containing unlabeled calpastatin domain I S241 (0-2 μM), porcine calpain 1 (0-1 μM), or rat calpain 2 C105A (0-1 μM) with C105A-eGFP (1 μM or 0.5 μM with calpain 1) and calpastatin domain I C148S241-AF546 (1 μM or 0.5 μM with calpain 1). Results are each from a single experiment.  

B) In a binding assay with calpastatin domain I C148S241-AF546 (1 μM) bound to C105A-eGFP (1 μM) in the presence of calcium (10 mM), unlabeled calpastatin domain I S241 was then added up to 13 μM to compete for binding of the calpastatin domain I C148S241-AF546.  

The ratio of acceptor emission over donor emission was determined at 0 μM (light blue) and 13 μM (blue) unlabeled calpastatin (n=1).
response to calcium. These results confirm calpastatin domain I and both calpains 1 and 2 do compete for binding and indicate their presence in situ could decrease the FRET signal. In a similar assay unlabeled calpastatin domain I S241 was added to prebound calpastatin domain I C148S241-AF546 and C105A-eGFP (figure 3.11 B). Up to 13 μM unlabeled calpastatin domain I was unable to displace the bound calpastatin domain I C148S241-AF546 from C105A-eGFP. This argues that calpastatin may have a slow off rate of binding to calpain 2.

Endogenous calpain substrates will also be present in cells. Binding of these substrates in situ may also compete for acceptor-calpastatin binding to C105A-eGFP and potentially decrease the FRET signal. In order to investigate the potential for competitive binding, one of calpain’s endogenous substrates the cytoplasmic tail (CT) of β1 integrin and an in vitro substrate, α-casein, were used as competitors for the binding of calpastatin domain I C148S241-AF546 to C105A-eGFP. The calcium dependent increase in $\frac{E_{m_A}}{E_{m_D}}$ due to the binding of calpastatin domain I C148S241-AF546 to C105A-eGFP was determined in the presence of partially purified β1 integrin-CT or α-casein. Up to 20 μM partially purified β1 integrin-CT or 20 μM α-casein did not affect the increase in FRET ratio upon calcium addition (figure 3.12 A). In similar assays β1 integrin or α-casein were preincubated with C105A-eGFP in the presence of calcium to favor binding of the substrates. Addition of calpastatin domain I C148S241-AF546 revealed no measurable change in the ratio (data not shown) in contrast to the effect observed with unlabeled calpastatin. Thus neither substrate tested competed for the FRET signal. To verify that calpain is actually binding the β1 integrin-CT, WT calpain 2 was allowed to proteolyze the partially purified β1 integrin-CT. As early as 10 minutes calpain cleaved the β1 integrin demonstrating that calpain must be binding the substrate (figure 3.12 B). Although casein is not an endogenous substrate it is a very good in vitro substrate for calpain 2. The inability of even α-casein to compete for acceptor-calpastatin binding indicates many substrates may not pose a problem as competitors in situ.
Figure 3.12  Calpain Substrates do not Compete with Calpastatin Domain 1 C146S241-AF546 Binding to C105A-eGFP

To assess the ability of endogenous substrates to compete for the binding of C105A-eGFP to calpastatin domain 1 C146S241-AF546, partially purified β1 integrin cytoplasmic tail or α-casein were used as competitors. A) The change in ratio upon addition of calcium (10 mM) was determined for a series of binding assays containing partially purified β1 integrin cytoplasmic tail or α-casein (0-20 µM) with C105A-eGFP (1 µM) and calpastatin domain I C146S241-AF546 (1 µM). Competitor concentrations are 0 µM (cyan), 1 µM (blue), 10 µM (red), and 20 µM (yellow). Results are each from a single assay. B) To verify calpain's ability to bind the substrate the β1 integrin cytoplasmic tail was proteolyzed by WT calpain 2. Recombinant rat calpain 2 (50 ng/µL) was incubated with the partially purified β1 integrin cytoplasmic tail (0.5 µg/µL) in the presence of calcium (10 mM) at 22°C. Aliquots were treated with SDS sample buffer after 0, 10, and 60 minutes and analyzed by electrophoresis. Migration of molecular weight markers (USB) is indicated in kDa. Band corresponding to β1 integrin is indicated by an arrow. Gel pictured is 10% acrylamide and stained with Coomassie blue-amido black.
Chapter 4  DISCUSSION

Two strategies were tested to develop a sensor for detecting active calpain. The strategy utilizing calpain 2 modified with a donor fluorophore at the carboxy-terminus of domain IV to donate energy to calpastatin domain I modified with an amino-terminal acceptor fluorophore did generate FRET. Two different donor fluorophores were utilized, FlAsH and eGFP. The biarsenic dye FlAsH is a derivative of fluorescein where as eGFP is an expressible fluorogenic protein derived from jelly fish. FlAsH is a small dye where as eGFP consists of a chromophore protected by a bulky β-barrel. The location of the chromophore in the barrel of eGFP adds ~30 Å to the distance between the fluorophores (53). The actual distance between eGFP and the acceptor fluorophore would shift from ~31 Å to ~61 Å. This increase in fluorophore distance is closer to the maximum $R_0$ for many fluorophore pairs (60 Å). Thus the smaller size of FlAsH is predicted to allow it to maintain a closer distance to the bound acceptor fluorophores increasing the efficiency in energy transfer.

There is also a difference in the emission spectra of the two donor fluorophores. FlAsH’s emission peak is shifted approximately 20 nm longer than that of eGFP, 528 nm vs. 508 nm. The shifted donor emission would allow greater overlap of the donor emission and acceptor absorbance peaks generating more efficient transfer of energy from FlAsH than eGFP. In fact, there was a greater calcium dependent increase in ratio for FlAsH as donor vs. eGFP (figure 3.8). Also, the acceptor direct excitation seemed closer to the FRET acceptor emission for eGFP as donor vs. FlAsH (figure 3.6). These results support the speculation that FlAsH may transfer energy more efficiently to TMR and AF546 than eGFP.

Orientation and distance of the fluorophore pair relative to each other are important factors for the efficiency of energy transfer. Preferentially positioning the acceptor fluorophore at
three different locations near the amino-terminus of calpastatin domain I (C_{142}, C_{148}, or C_{151}) slightly altered the distance (2-10 Å) and potentially the orientation between the fluorophore pairs. There was not a superior acceptor fluorophore position out of the three tested (figure 3.8). Both TMR and AF546 have very similar excitation and emission spectra however Alexa Fluor dyes are among the brightest and most photostable fluorescent dyes (Alexa Fluor Dyes Invitrogen). Despite the advantages of the AF546 neither acceptor fluorophore proved superior compared to the other in these studies.

The fluorogenically modified calpastatin peptides, despite containing a single cysteine residue, were modified with 2 or more fluorophores per peptide based on the determined stoichiometry of peptide labeling. Although more selective for thiols, maleimides have the capacity to interact with the ε amino group of lysines at neutral pH and/or at high fluorophore-maleimide concentrations (4). The fluorogenic peptide conjugation assays were performed at pH 7.0 with a 13 fold molar excess of fluorophore-maleimide to peptide (40-80 μM). Calpastatin domain I contains 19 lysine residues with 11 in subdomain A and 8 in subdomain C that may have been nonspecifically conjugated under the assay conditions. Modification of calpastatin domain I S_{241}, a peptide containing no cysteine residue, established that approximately half of these peptides were nonspecifically coupled with TMR. In order to bias selectivity of modification towards cysteine residues the pH of the reaction was lowered to 6.0 and the incubation time for modification was decreased to thirty minutes. Neither of these changes decreased the estimated stoichiometry of TMR labeling of calpastatin domain I S_{241} (data not shown).

When the AF546/TMR modified calpastatin domain I S_{241} peptides served as acceptor to C_{105A}-eGFP in binding assays the increase in the E_{mA}/E_{mD} ratio was approximately four fold lower than that of the peptides modified at an amino-terminal cysteine (figure 3.8 B). There are two possible contributing factors for the lack of detectable energy transfer from eGFP. First,
approximately half the bound calpastatin domain I S_{241} possess a TMR to accept energy transfer from the eGFP lowering the detectable signal. Also, the position of acceptor fluorophore on the calpastatin peptide is important to the efficiency of energy transfer. The calpastatin domain I peptides encoding a cysteine are all preferentially labeled near the amino-terminus with an acceptor fluorophore. The position of modification of the nonspecifically labeled domain I S_{241} is undetermined and likely sporadic. The nonspecifically modified peptides are likely a population with mixed acceptor fluorophore localized along the length of the peptide. It is predicted that the positioning of the acceptor fluorophore at the amino-terminus of bound calpastatin domain I allowed for more efficient transfer from eGFP than distal locations. A construct expressing calpastatin domain I C_{241} was created during the subcloning process but was not expressed or purified. Modification of this peptide would specifically direct acceptor fluorophore labeling to the carboxy-terminus of calpastatin domain I. Calpastatin domain I C_{241}-TMR and domain I C_{241}-AF546 could serve as acceptors to C_{105A}-eGFP in binding assays to demonstrate if the amino-terminal localization of acceptor fluorophore is essential for efficient energy transfer. A detectable signal would reveal that an acceptor fluorophore preferentially placed towards the carboxy-terminus of calpastatin domain I can also accept energy transfer from eGFP at the carboxy-terminus of calpain domain IV.

Two different approaches were utilized to assess energy transfer from the donor on the carboxy-terminus of calpain 2 domain IV to the acceptor on the amino-terminus of bound calpastatin domain I. The two assay strategies utilized either increasing acceptor-calpastatin concentration or increasing calcium concentration. The assays employing increasing acceptor-calpastatin displayed high direct excitation of the acceptor. The contribution of direct excitation of the acceptor fluorophore to the acceptor emission prevented the \( \frac{E_{m_A}}{E_{m_D}} \) ratio from demonstrating saturation of binding of the C_{105A}-eGFP and C_{105A}-FlAsH. In addition, the acceptor emission spectra shifted as the acceptor-calpastatin concentration increased. This shift
affected the $E_{mA}/E_{mD}$ ratio calculated from the relative fluorescent units at the peak of the acceptor emission spectra at the low acceptor-calpastatin concentrations (1-4 μM). For this reason the area under the donor and acceptor emission peaks was used to calculate ratios for these assays. The shift in acceptor emission peak was not a problem however when donor-C105A and acceptor-calpastatin concentrations were used at a fixed concentration. Thus the assays utilizing 1 μM C105A-eGFP, 2 μM acceptor-calpastatin and increasing calcium concentration circumvented the effects of high acceptor-calpastatin concentration.

Ultimately, this tool will be of the most use in cells to demonstrate spatial and temporal activation of calpain. As expected the binding of acceptor-calpastatin to C105A-eGFP was specific enough to work in the crude environment of an E. coli cell lysate. The assays using calpain 1 as a competitor indicate calpastatin domain I C148S241-AF546 also binds calpain 1. Utilizing a crude cell lysate containing calpain 1-eGFP fusion protein could prove the ability of calpain 1 to bind calpastatin domain I C148S241-AF546 during in vitro binding assays. Employing cells expressing either calpain 2-eGFP or calpain 1-eGFP, a comparison could be made of the potential differential spatial and temporal subcellular localization of the two classical calpain isoforms. The assays using calpastatin domain I S241, calpain 1, or calpain 2 C105A as competitors also demonstrated there would be some signal interference due to endogenous calpastatin and calpain. The levels of endogenous calpain and calpastatin expression are different between cell types. Human platelet cells display calpain 1 and calpastatin but little to no calpain 2. Bovine cardiac muscle cells contain more calpain 2 than calpain 1 and calpastatin in excess of both proteases (18, 57). Also, full length calpastatin contains four inhibitory domains to bind up to four calpain molecules. Therefore the potential level of signal interference due to these three endogenous proteins will depend on the type of cells employed in the study. For example, the Capn4-/- murine fibroblasts deficient in the calpain small subunit demonstrate a lack of calpain activity by casein zymography (13). Alternatively, siRNA have been used to knock down calpain
1 in human breast cancer cells (MCF-7) which lack expression of calpain 2 (62). Thus interference could be reduced by selecting a cell type low or deficient in endogenous classical calpains and/or calpastatin.

Of further interest was the potential for endogenous calpain substrates to compete for the binding of acceptor-calpastatin to C105A-eGFP. As demonstrated by competition using the endogenous substrate β1 integrin-CT and the in vitro substrate α-casein, it appears that most substrates should not inflict this complication. This is likely due to calpastatin's high affinity for calpain (k_0=3 nM) (42). Calpastatin's ability to displace bound substrate could prevent calpain from cleaving substrate in the event of unwanted activation. This may be an important protective role for calpastatin in vivo.

In situ overexpression of calpain 2-eGFP and microinjection of calpastatin domain I-acceptor will be necessary to utilize this strategy in cells. Increased calpain activity is associated with photoreceptor degeneration, failure of cell-cycle arrest in mammary and lung carcinoma cells, amyloid plaque and neurofibrillary tangle formation in Alzheimer's disease, and apoptosis (26, 27, 45, 46). Alternatively, increased calpastatin expression is associated with reduced formation of lamella and altered gene expression (7). Depending on the cell type being used overexpressing calpain and injection of calpastatin domain I in cells could have adverse effects upon calpain activation. To avoid such issues the C105A calpain 2 inactive mutant could be used in place of WT calpain 2. If observing calpain dependent focal adhesion turnover in fibroblasts, added calpastatin domain I-acceptor may inhibit endogenous calpain and prevent the expected adhesion turnover (16). Acceptor-calpastatin domain I could be injected at different concentrations to find one that does not inhibit adhesion turnover but still allows signal detection. A similar probe using fluorogenic calpastatin subdomain A and C peptides to bind calpain would be capable of detecting endogenous calpain without inhibition. Neither of the calpastatin subdomain peptides contain a intact subdomain B region. Disrupting the subdomain B region
diminishes its inhibition (1). Thus injection of the subdomain A and C peptides would not inhibit endogenous calpains allowing for detection of subcellular activation during cellular processes such as focal adhesion turnover.

The binding of calpastatin to calpain has been reported to occur in the absence of calcium based on data obtained by co-immunoprecipitation (38). This result contradicts the long established calcium dependent binding interaction of calpastatin to calpain that was demonstrated by several methods (8, 63). The cooperative increase in the $\frac{E_{m_A}}{E_{m_D}}$ ratio directly demonstrates binding between calpain and calpastatin in response to calcium and a reversal of binding in response to the calcium chelator EGTA (figure 3.7). Immunoprecipitate of $\text{C}_{105}A$-eGFP utilizing an antibody for eGFP co-precipitates calpastatin domain I only in the presence of calcium (Croall unpublished data). These data do not support a calcium independent association between calpastatin and calpain.

Unfortunately, subdomain C-AF568 did not demonstrate energy transfer to subdomain A-AF647 in vitro. Similarly, $\text{C}_{105}A$-eGFP did not demonstrate energy transfer to subdomain C-AF546 despite an approximately 31 Å distance between the fluorophores when bound. The lack of signal observed with the fluorogenic subdomain C peptides may be due to multiple problems including fluorophore distance, unfavorable fluorophore orientation and/or lack of binding. The distance from the carboxy-terminus of domain IV of calpain to the carboxy-terminus of bound calpastatin domain I subdomain C is the same distance estimated to the amino-terminus of bound subdomain A. Thus fluorophore distance is unlikely to explain the inability to detect energy transfer from $\text{C}_{105}A$-eGFP to subdomain C-AF546 peptides. The amino-terminus of calpain bound calpastatin domain I subdomain A is estimated to be 50 Å from the carboxy-terminus of calpain bound calpastatin domain I subdomain C. This distance is close to the maximum $R_e$ for many fluorophore pairs (60 Å). A perpendicular fluorophore orientation results in a zero net rate of energy transfer. If bound subdomain C-AF546 is not in the proper orientation energy will not
be transferred from eGFP at the carboxy-terminus of calpain domain IV despite the distance. Potentially adding to the problem the acceptor fluorophore AF647 demonstrated decreased intensity compared to TMR, AF546, and AF568. This decreases the overlap of the AF568 emission spectra with the AF647 excitation spectra decreasing the potential for energy transfer. Thus there could be a combined problem of fluorophore distance, orientation and acceptor fluorophore intensity for detecting the binding of subdomain C-AF568 and subdomain A-AF647 to calpain.

There is concern whether the acceptor modified calpastatin domain I subdomain C peptides are binding calpain. Calpastatin domain I subdomain C L235 and F239 are the main residues binding the two hydrophobic pockets of calpain domain VI. Positions 237 and 241 of calpastatin subdomain C are very close to these important residues. Fluorogenic modification of subdomain C at positions C237 and C241 may sterically hinder binding of subdomain C to calpain domain VI. Calpastatin subdomain C S241C246 and S241C256 however direct modification to locations distal to the identified subdomain C calpain domain VI interaction sites. Thus calpastatin subdomain C labeled at position 246 or 256 should not affect subdomain C’s ability to bind calpain. There is evidence however that the calpain heterodimer in the presence of calcium and in the absence of substrate or calpastatin is unstable and dissociates. The small subunit may precipitate under such conditions, thus calpain would no longer be a heterodimer containing domain VI for calpastatin subdomain C to bind. The subdomain A and C peptides do not constitute a whole domain and binding may not be sufficient to maintain calpain stability. This could also explain the inability to detect energy transfer from the eGFP on the carboxy-terminus of calpain domain IV to subdomain C-AF546.

A series of future experiments are proposed to investigate whether the problem is due to acceptor fluorophore position on calpastatin or subunit dissociation. First, a construct expressing calpastatin domain I S241C246 could be engineered by mutating T246 to encode a cysteine in the
construct containing the calpastatin domain I $S_{241}$ sequence. Purified calpastatin domain I $S_{241}C_{246}$ modified with AF546 could serve as acceptor to $C_{105}$A-eGFP to provide information about subdomain C binding. Calpastatin domain I $S_{241}C_{246}$-AF546 contains the subdomain A and B binding sites. If small subunit dissociation is the issue the presence of the subdomain A and B binding sites should increase heterodimer stability. Thus there should be a FRET signal with the acceptor fluorophore at position 246. Second, it has been shown that $\alpha$-casein does not compete for binding of calpastatin domain I $C_{148}S_{241}$-AF546 to $C_{105}$A-eGFP. Similar to the effect of calpastatin, calpain substrates are expected to stabilize the heterodimer in the presence of calcium (44). Calpastatin subdomain C $C_{246}S_{241}$-AF546 could serve as acceptor to $C_{105}$A-eGFP in the presence of $\alpha$-casein to prevent small subunit dissociation. The detection of energy transfer upon calcium addition in these assays would support the occurrence of subunit dissociation in the absence of $\alpha$-casein. If this proves to be true, the assays utilizing calpastatin subdomain C-AF568 as donor to subdomain A-AF647 should be repeated with the inclusion of $\alpha$-casein.

Last, the binding of a calpastatin domain to calpain orients the amino-terminus of subdomain A inverted to that of subdomain C (figure 1.3). The orientation of the amino-terminal fluorophore/cysteine positions of domain I (subdomain A) to calpain domain IV carboxy-terminus may be more comparable to amino-terminal positions of subdomain C. Residues 237, 241, 246, and 256 used to date as fluorophore modifying locations on calpastatin subdomain C peptides are at the carboxy-terminus. Specifically placing a fluorophore at the amino-terminus of subdomain C may put the fluorophore in a more favorable orientation for energy transfer. The amino-terminus of calpastatin subdomain C is estimated to be ~37 Å from the carboxy-terminus of calpain domain IV and ~51 Å from the amino-terminus of calpastatin domain I (subdomain A). Calpastatin residue $S_{223}$ is at the amino-terminus of subdomain C but not too close to the key residues responsible for binding calpain domain VI. Constructs encoding calpastatin domain I $S_{241}$ and/or subdomain C $S_{241}$ could be mutated to encode a cysteine at position 223. Calpastatin
domain I $C_{223S241}$ and subdomain C $C_{223S241}$ could be modified with AF546 to serve as acceptors to $C_{105A-eGFP}$ in binding assays. In the absence of positive results from the experiments above, the detection of energy transfer in these assays would support acceptor fluorophore orientation at the carboxy-terminus of subdomain C as unfavorable. If these assays demonstrate a FRET signal, calpastatin subdomain C $C_{233S241}$ could then be modified with AF568 to serve as donor to the calpastatin subdomain A-AF647 peptides to detect binding to unlabeled calpain. An inability to detect energy transfer even in the presence of $\alpha$-casein would suggest there is insufficient spectral overlap between the AF568 and AF647 fluorophore pair to allow energy transfer possibly due to the weak AF647 signal as mentioned above. A fluorophore pair with greater spectral overlap may remedy this problem such as AF546 as donor to Alexa Fluor 594.

A novel method to detect endogenous calpastatin by FRET was recently described (19). The purpose of the study was to detect endogenous calpastatin in a heated meat extract using AF546 modified mouse anti-calpastatin IgG as donor to AF594 modified calpain 1 that could be brought together when bound to calpastatin. However, the antibody and calpain 1 were randomly modified with fluorophores and the distance between the antibody and calpain binding site on calpastatin was not defined. Thus the distance between the fluorophores of the bound fluorogenic proteins of the sensor was unknown. Despite the flaws in this design, the ability of the FRET sensor to detect calpastatin in the meat extract was demonstrated by a reduction in the $Em_D/Em_A$ ratio in response to calpastatin addition (120-180 ng/mL). Binding of the sensor to calpastatin was implied by these competition studies. To verify the sensor is binding calpastatin in a calcium dependent manner, the change in the $Em_D/Em_A$ ratio in response to increasing calcium concentration could be monitored in the presence of several concentrations of purified calpastatin. To ensure the signal is a detection of sensor binding to calpastatin, this experiment could be performed in the absence of calpastatin. These results could validate the ability of this strategy to generate a FRET signal in the presence of calpastatin despite the undefined binding interaction.
and unknown fluorophore separation. In contrast, the strategy detecting the binding of donor-calpain and acceptor-calpastatin, as developed in this thesis, has the advantage of a well characterized binding interaction and the ability to estimate the distance between the fluorophore pair of the bound fluorogenic calpain and calpastatin. However, it may be possible to detect endogenous calpain using an AF546 modified anti-calpain antibody (AF546-Ab) to serve as donor to AF594 modified calpastatin domain I. Depending on the specificity of the antibody it may also be possible to visualize activation of the different classical calpain isoforms. Success of this sensor would depend on the anti-calpain antibody binding to a region that does not sterically hinder binding of calpastatin domain I to calpain and to bind a region that is within 60 Å distance from the AF594 on the calpastatin domain I. In the absence of a pair of fluorogenic calpastatin subdomain peptides that bind calpain to generate FRET, application of an AF546-Ab/AF594-calpastatin could be an alternative strategy to detect endogenous calpain.

Future studies may also be aimed at developing new designs of a FRET probe to detect active endogenous calpain. One design would be to develop a probe that will detect a loss of FRET due to cleavage specifically by calpain. This has been previously attempted by expressing the α-spectrin calpain cleavage site between eCFP and eYFP with and without an amino terminal PDZ-binding site (52, 59). The PDZ-binding site directed localization of the fusion protein to areas of synaptic calpain activation in hippocampal neurons. This generated positive results biased to demonstrate calpain activation specifically at these locations in cells. This sensor was also expressed in mouse tibialis anterior muscles without the PDZ-binding site to demonstrate calpain activation due to calcium injection. As previously mentioned, α-spectrin can also be cleaved by caspase-3 indicating the loss of FRET signal for the eCFP/α-spectrin/eYFP sensor is not exclusively diagnostic for calpain activation. To overcome this limitation the β1 integrin-CT calpain cleavage site could be tethered to calpastatin subdomain A and expressed between CFP and YFP. The unstructured nature of β1 integrin-CT and subdomain A would allow FRET
between CFP and YFP in the absence of cleavage (43). Upon binding calpain, cleavage of the β1 integrin-CT would release a fluorophore allowing detection of a loss of FRET signal. The ability of a calpain molecule to cleave multiple probes increases the sensitivity of the signal for the cleavage probe compared to the binding probe. Importantly, the fusion of a calpastatin binding region and calpain cleavage site could improve the specificity of the loss of FRET sensor.

Application of the calpain-eGFP and calpastatin domain I-AF546 to in situ studies may provide a useful tool for detecting where and when calpain is active during both pathological and physiological cellular processes. To accomplish this, an optimum concentration of acceptor-calpastatin for injection into a cell line expressing calpain-eGFP will need to be established. Unfortunately competition due to both endogenous calpains and calpastatin will be concern for this strategy in cells. The strategy to detect active calpain using the binding of fluorophore modified calpastatin subdomain A and C peptides would have the advantage of detecting endogenous calpain without inhibition. Upcoming studies are therefore aimed to continue the development of probes that will detect activation of endogenous calpain in situ by exploiting the calpain-calpastatin binding interaction.
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