The first goal of this study was to understand the role of calpains in skeletal muscle protein degradation in cultured cells. We have developed a genetic approach to inhibit endogenous calpain activity through over-expressing dominant negative m-calpain (DN), antisense m-calpain (AS) and calpastatin inhibitory domain (CID). We observed that, under conditions of accelerated degradation (serum withdrawal), inhibition of m-calpain through DN-m-calpain over-expression caused a 30% inhibition of total protein degradation whereas CID over-expression reduced degradation by 63%. These constructs did not significantly affect degradation in the presence of serum. These data indicate that calpains participate in the accelerated degradation associated with serum withdrawal. Inhibition of calpain also stabilized nebulin, a major structural protein of the sarcomere. These observations indicate that calpains play significant roles in muscle protein turnover. Finally, over-expression of antisense m-calpain caused a transient reduction in m-calpain concentration after which normal m-calpain concentration was quickly re-established. These observations indicate that m-calpain is a short half-life protein in muscle cells.

The second goal of this study is to investigate the role of calpain in the mediation of PARP protein level in differentiating myoblasts. Poly(ADP-ribosyl)ation, catalyzed by PARP, is involved in various physiological events, such as DNA excision repair, DNA recombination, DNA replication, cell differentiation, cell growth and transformation, and apoptosis. A protease participating in PARP turnover could be a significant regulator to
the events which PARP is involved. A relationship between apoptosis and myofibrillar protein degradation via a common protease might suggest the basis for muscle wasting and atrophy which characterize in many muscle diseases. We established a genetic approach to inhibit endogenous calpain activity through over-expressing calpastatin inhibitory domain (CID). We observed that (1) inhibition of calpain activity increased PARP concentration when post-confluent myoblasts were cultured with 2 % HS medium, an inducer of differentiation and (2) inhibition of calpain activity prevented PARP degradation induced by A23187 and etoposide in differentiating myoblasts. These data demonstrate that calpain is involved in regulation of PARP in cultured cells.
Doctor of Philosophy thesis of Jing Huang presented on April 13, 1998

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Chairman of Genetics Program

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Jing Huang, Author
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I would like to thank Drs. Mathews, Hu, Whanger, Froman and Slabaugh for serving on my program committee. On many occasions all provides an outside perspective or opinion which helped clear my way to completion of this project.

Viola and Laura are deeply thanked for their unlimited support and great friendship.

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DEDICATION

This Doctoral Dissertation is dedicated to my parents, Jiaxi and Caixiang, my husband, Junru, and my daughter, Shiyuan. I couldn't have done this without your tolerance and loving support. Thank you for everything.
Skeletal muscle is a major tissue of the body, accounting for forty percent of total body weight in normal adult animals. The accumulation of muscle tissue or muscle growth depends on both the rate of muscle protein synthesis and the rate of muscle protein degradation. It is clear that muscle proteins turn over metabolically with half lives ranging from 2 to 20 days (1) or 5 to 10 % per day. The rate of muscle protein degradation can vary over a wide range in response to physiological demand (2,3). Therefore, variations in rate of muscle protein degradation could be a primary cause in regulating the rate of muscle growth among domestic animals and could, in some instances, be more important than the rate of muscle synthesis in such regulation. It has been estimated that 15 to 25 % of the food ingested by domestic animals is used to replace muscle protein that is degraded during metabolic turnover (4). Therefore, decreasing the rate of muscle protein degradation would decrease the amount of energy needed for protein replacement and should lead directly to increases in efficiency with which animals convert ingested nutrients into edible muscle. Dystrophic and atrophic muscle, those formation involves dramatic increases in muscle protein degradation, are observed in many types of muscle diseases. Little is known, however, about the mechanism of muscle protein degradation and the nature of the proteolytic enzymes responsible for this turnover. Consequently, it is important to learn what enzymes are involved in intracellular degradation of muscle proteins.

Muscle diseases was accompanied by a process termed apoptosis (programmed cell death). Large decreases in protein degradation have been observed as a major change in many types of muscle diseases. Apoptosis is poorly understood in skeletal muscle but
some evidence from other tissues indicates that the enzymes which degrade to bulk of muscle protein (calpains) are responsible for digestion of some proteins involved in apoptosis (e.g., poly (ADP-ribose) polymerase; PARP).

Poly (ADP-ribosyl)ation has been suggested to be involved in various physiological events, including DNA excision repair, DNA recombination, DNA replication, cell differentiation, cell growth and transformation (5), and carcinogeneses in vivo (5-7). Inhibition of poly(ADP-ribose) formation through control of poly(ADP-ribose) polymerase (PARP) activity prevents several types of apoptosis (programmed cell death; 8-10). PARP cleavage is generally a hallmark of apoptosis. However, the enzyme responsible for PARP cleavage has proved to be unexpectedly controversial. Both Nicholson’s and Lazebnik’s groups have reported that caspase-1 (interleukin 1-β-converting enzyme, ICE) itself is unable to cleave PARP (11,12) even when the protease was present in a 500-fold molar excess (11). Recent studies by Gu et al. (13), in contrast, reported that PARP is cleaved by caspase-1, caspase-4 (TX, an ICE homologue), or Nedd2 (an ICE homologue) when these proteases are present in excess compared to substrate. A protease, caspase-3 (CPP32/apopain, an ICE homologue), which cleaves PARP during activation of apoptosis has been independently identified by three labs (11,14,15). More recently, however, it was reported that PARP can be processed by calpain in vitro (16). Consequently, it is interesting to know if calpain is involved in the degradation of PARP in living cells.

It is of considerable interest to assess the role of calpain in apoptosis since this process has been detected in some muscle diseases. It has been reported that degenerating fibers in the mdx mouse, a animal model of Duchenne type muscular dystrophy (DMD), show an increase in membrane permeability and undergo apoptosis (87). Further, apoptosis may be involved in early events associated with muscle differentiation (56). Here, knowledge of the role of calpain in apoptosis will provide further understanding of muscle disease and growth.
Calpain and muscle protein degradation

Calpain and calpain family: Calpain (EC 3.4.22.17) is a Ca\(^{++}\)-dependent cysteine protease. A Ca\(^{++}\)-dependent neutral proteinase in rat brain was first reported in the early sixties (17,18) and named as calpain several years later. Calpain was first purified to homogeneity by Imahori's group (19) in 1978. The first calpain large subunit cDNA was cloned in 1984 and named as chicken u/m-calpain (20). Since then, various types of calpain subunits and their homologs have been identified and their primary structures determined by cDNA cloning. In the calpain family (mammals), two ubiquitous calpain isoforms are well-characterized (\(\mu\)- and m-calpain), and at least 5 tissue-specific isoforms have been identified (21). Various "atypical" homologies of calpain are also being reported in lower organisms, such as insects, nematodes, fungi and yeast. All the members in calpain family share a similar activity domain, similar to other cysteine proteases such as papain and cathepsin B, H, and L.

Calpain structure and regulation: \(\mu\)- and m-calpain (conventional calpains) are heterodimeric proteins, including one large subunit (80 kDa) and one small subunit (30 kDa). The large subunit can be sub-divided into four domains: a propeptide domain (I), an activity domain (II), a regulatory domain (III), and a calcium-binding domain (IV). The small subunit includes a calcium-binding domain (IV') and a glycine-rich domain (V). Calpain activity in living cells is highly regulated. Calcium is essential for calpain activity (conventional type). Both autolysis and phospholipids increase the sensitivity of calpain to calcium. Autolysis of calpain occurs at the N-terminus of both the large subunit (domain I) and small subunit (domain V). Bovine skeletal muscle calpain autolyzes into 78 kDa (m-calpain) or 76 kDa (\(\mu\)-calpain) large subunits and an 18 kDa small subunit for both \(\mu\)- and m-calpain (22). The calcium requirement for calpain activity is reduced by the autolysis and by the presence of phospholipids (phosphatidylinositol-4,5-bisphosphate \(_{\text{PIP2}}\) > phosphatidylinositol-4-phosphate \(_{\text{PIP}}\) > phosphatidylinositol \(_{\text{P}}\)). Autolyzed \(\mu\)-calpain, in bovine skeletal muscle, requires 0.6 \(\mu\)M for half-maximal activity; preautolyzed \(\mu\)-calpain requires 7.1 \(\mu\)M; autolyzed m-calpain requires 180 \(\mu\)M Ca\(^{++}\); and preautolyzed m-calpain
requires 1 mM Ca\(^{++}\). In the presence of PIP\(_2\), \(\mu\)-calpain is active at 100 nM Ca\(^{++}\) whereas m-calpain requires about 10 \(\mu\)M, or higher, Ca\(^{++}\) (23). The regulation of calpain activity is also affected by calpastatin, the endogenous calpain inhibitor. It is present in all cells that contain calpain. Calpastatin exists predominantly as a polypeptide of 713-718 residues and inhibits 1-10 mol of calpain/mol of calpastatin. The inhibitory activity is heat-stable, competitive, specific and reversible, and can be attributed to any of 4 homologous functional repeats. Although the intact calpastatin may have additional interactions with calpain, the central consensus sequence (TIPPEYR) of each repeat has been demonstrated to be necessary and sufficient for specific inhibition of calpain (24-29).

**Involvement of calpain in physiological events:** Calpain is a multifunctional protease. Its processing, instead of digestive, activity determines its significant role in a variety of physiological events. Calpain substrates (23) include cytoskeletal proteins, actin-binding proteins (fodrin, spectrin, talin, filamin, etc), microtubule-associated proteins; membrane proteins such as growth factor receptors; enzymes such as kinases, phosphatases and phospholipases; cytokines (interleukin 12); transcription factors (Fos, Jun); tumor suppressor p53 (21,30); PARP (16); transforming growth factor-\(\beta\) in hepatocytes (TGF-\(\beta\)) (31). This list is growing quickly. Calpains have been suggested to be involved in various physiological and pathological events, including myoblast differentiation (32), muscle protein degradation (23, 34-38), platelet activation (39-41), muscular dystrophy- or atrophy- associated diseases (42,43), brain-associated diseases (23), such as ischaemia and Alzheimer's disease, and apoptosis (44-47).

**Components, structure and degradation of skeletal muscle:** The muscle is made up of bundles (fascicles) of fibers, each bundle being lined by perimysial connective tissue. The most obvious structures within the muscle fiber are the myofibrils, which are the units responsible for contraction and relaxation of the fiber. Each fiber contains up to 8,000 myofibrils and contains two types of protein filaments, actin and myosin. The two types of filaments support each other through cross-bridges and are themselves supported by other specialized structures. Repeating units of a myofibril are named sarcomeres. These are composed of an array of overlapping thick (myosin) and thin (actin) filaments
between two adjacent Z discs. The plate-like region of a muscle sarcomere is the Z disk. This consists of a latticework from which the actin thin filament originates. Each of the myosin filaments is surrounded by a hexagonal lattice of actin filaments. The actin filaments contain, in addition to actin, two regulatory proteins, troponin and tropomyosin, and also a strengthening protein, nebulin. The Z disk is composed mainly of the proteins \( \alpha \)-actinin, desmin, vimentin, and synemin, which form intermediate filaments that are wrapped around the disk and also link adjacent discs together in the longitudinal and transverse axes. Degradation of myofibrils involves two steps. The first step is to dissemble myofibril, initiated from Z disk. The second step is to digest individual myofibrillar proteins into amino acids. The first step is a rate-limiting step. Therefore, to identify the protease which mediates this step will facilitate controlling of myofibrillar protein turnover and understanding of the mechanism of myofibril degradation (48).

**Previous studies on the involvement of calpain in myofibril degradation:**

Evidence that calpains mediate disassembly of the myofibrillar apparatus is quite compelling. Calpain initiates digestion of individual myofibrillar proteins including desmin, filament, C-protein, tropomyosin, troponin T, troponin I, titin, nebulin, vimentin, gelsolin and vinculin (34,35,49-56). However, calpains do not degrade \( \alpha \)-actin, \( \alpha \)-actinin or myosin heavy chain (33,34). Calpains tend to be concentrated in the Z-disk (36), the site where disassembly begins (57). Calpains are activated by calcium and treatment of purified myofibrils with calcium causes rapid and complete loss of the Z-disk (58). Calpains are also activated in conditions of muscle wasting. Increased calpain activity in muscle has been reported in vitamin E deficiency (42), Duchenne dystrophy (59-62) and fasting (43). Calpain mRNA concentrations are increased markedly during fasting (37). The time course for changes in calpain activity corresponds to morphological changes in muscle (63). Based on these data, Goll et al (57) and others (33,34,37,43,57,62-66) have proposed that calpain plays a significant role in myofibrillar protein degradation, especially in the disassembly of the myofibril during early stages of turnover. Degradation of the Z-disk by calpain is proposed to cause disordering of the three-dimensional architecture of...
the thick and thin filaments in muscle, thus making the exposed myofibril more susceptible to proteolytic attack by other, less specific proteases (63).

Limitations to the belief that calpains cause myofibrillar protein disassembly are that the calcium concentrations required for activity are much higher than those which are physiologically attainable. And, many reports have shown that the proteasome (multicatalytic protease) supersedes the role of calpain in myofibrillar protein degradation (67-72). The proteasome, a large ubiquitous ATP- and ubiquitin-dependent proteolytic system, is able to degrade actin and myosin in vitro (68). A recent study by Solomon et al (68) indicated that the proteasome degrades intact monomeric myofibrillar proteins but not the same proteins when they were associated with other myofibrillar proteins.

Evidence of calpain involvement in apoptosis

Several lines of evidence suggest the involvement of calpain in apoptosis. In thymocytes, calpain inhibitors PD150606 (10 μM), E-64d (300 μM), MDL28170 and calpain inhibitor I (20 μM), prevent dexamethasone-induced apoptosis, but not valinomycin- and heat-shock-mediated apoptosis (44). In primary cultures of hepatocyte, calpain inhibitors (I and II) attenuated TGF-β-induced apoptosis (45). Calpain inhibitors also blocked apoptosis in murine T cell lymphocytes triggered by engagement of the T cell receptor, but not apoptosis induced by steroids (46). Finally, calpain inhibitors blocked activation-induced apoptosis of HIV-infected T lymphocytes and restored effective immune response in these cells in vitro (47).

Involvement and processing of poly(ADP-ribose) polymerase in physiological events

Poly(ADP-ribose) polymerase (PARP): PARP (EC 2.4.2.30) was first reported by Shizuta’s group (89). The purified enzyme is composed of three domains: DNA
binding, automodification and NAD-binding domains. PARP catalyzes the transfer of the ADP-ribose moiety from its substrate, NAD⁺, to a limited number of protein acceptors. The amino acid involved is glutamic acid and its carboxyl group is esterified through the OH-group on C-1 of ADP-ribose (73). DNA strand breaks are essential for PARP activation (74,75) and recognition by the enzyme is not sequence-specific (76,77). During DNA repair, PARP recognizes and binds to DNA strand breaks resulting in the activation of the enzyme and inaccessibility of the nick for DNA binding enzymes such as DNA polymerase (78). Automodification of PARP and poly(ADP-ribosylation) of the nucleosomal proteins included either in chromatin architecture or DNA metabolism by PARP then allows the decondensation of the DNA for access to the damaged region by repair enzymes (79). Automodification of PARP allows itself to be released from the DNA damage region and leads to the recondensation of DNA.

**Involvement of PARP in physiological events:** PARP has been suggested to be involved in various physiological events, including DNA excision repair, DNA recombination, DNA replication, cell differentiation, cell growth and transformation (5). More recently, studies in a variety of cell types undergoing spontaneous or induced apoptosis have demonstrated that the 116-kDa PARP molecule is cleaved to 36-kDa and 85-kDa fragments, which separate its DNA binding domain (36 kDa) from its NAD-binding catalytic domain (85 kDa), before or concomitant with degradation of nuclear DNA into nucleosomal fragments (80-82). Cleaved PARP is a hallmark of apoptosis in many mammalian cells (83,84), but not all cell types (85,86). Interestingly, the observation that PARP knockout mice have an apparently normal phenotype except for skin hyperplasia (85) raised questions about the role of this enzyme in normal cellular physiology.

**Proteases involved in PARP processing:** The protease(s) responsible for PARP cleavage has proven to be controversial. Two groups have reported that caspase-1 (ICE) itself is unable to cleave PARP (11,12) even when the protease was present in vast abundance over substrate (11). Recent studies by Gu et al. (13), in contrast, reported that PARP is cleaved by caspase-1, caspase-4 (TX), or Nedd2 when these proteases are
present in excess compared to substrate. A protease, caspase-3 (CPP32/apopain), which cleaves PARP during activation of apoptosis has been independently identified by three labs (11,14,15). Interestingly, however, it have been recently reported that PARP can also be processed by calpain in vitro (16).
References:


Chapter II

Role of Calpain in Skeletal Muscle Protein Degradation

Jing Huang and Neil E. Forsberg

Submitted to Proc. Natl. Acad. Sci.,
Abstract

The proteolytic system responsible for degrading myofibrillar proteins in skeletal muscle is not well-defined. The goal of this study was to evaluate the roles of the calpains (calcium-dependent proteases) in mediating myofibril degradation. Three strategies to regulate intracellular calpain activities were developed: over-expression of dominant-negative m-calpain, antisense m-calpain and over-expression of calpastatin inhibitory domain (CID). To express these constructs, L8 myoblast cell lines were prepared and transfected with LacSwitch plasmids which allowed for IPTG-dependent expression of a gene of interest. Dominant negative (DN) m-calpain and antisense m-calpain were expressed to specifically inhibit m-calpain-dependent degradation of muscle protein whereas the CID fragment was expected to inhibit all calpains. Inhibition of calpain activity stabilized fodrin (non-erythroid spectrin), a well-characterized calpain substrate. This indicated the strategies we developed to regulate calpain in living cells functioned as expected. Under conditions of accelerated degradation (serum withdrawal), inhibition of m-calpain with DN-m-calpain over-expression caused a 30% inhibition of total myotubular protein degradation whereas CID expression reduced degradation by 63%. These constructs did not significantly affect degradation in the presence of serum. These data indicate that calpains participate in the accelerated degradation associated with serum withdrawal. Inhibition of calpain also stabilized nebulin, a major structural protein of the sarcomere. These observations indicate that calpains play key roles in muscle protein turnover. Finally, over-expression of antisense m-calpain caused a transient reduction in m-calpain concentration after which normal m-calpain concentration was quickly re-established. These observations indicate that m-calpain is a short half-life protein in muscle cells.

Key words: calpain, muscle, protein degradation, fodrin, nebulin
Introduction

Skeletal muscle represents the largest pool of labile protein in the body. Within this pool, the myofibrillar proteins account for the bulk of skeletal muscle protein and, during diseases or starvation, these proteins are mobilized to nourish the entire organism. These adaptations may be critical to survival. However, there are instances where mobilization is excessive and wasting ensues. Muscle wasting occurs in cachexia (1,2), immobility (3), AIDS (4), endocrine disorders (5) and in genetic muscular dystrophies (6-9). Identification of the rate-limiting mechanism in muscle wasting may provide a target for intervention.

Two proteolytic systems have been studied with regard to their role in muscle protein wasting: calpains and the proteasome. Calpains are calcium-activated cysteine proteases which were originally identified in porcine muscle (10,11). Two ubiquitous isoforms are well-characterized (μ- and m-calpain) and several tissue-specific isoforms have also been reported (12). Calpains are regulated by a variety of factors. Ubiquitous calpains require the presence of a 30 kDa small subunit for activity (12), are activated by calcium and phospholipids (12-15) and are inhibited by calpastatin, a widely-distributed calpain-specific inhibitor (16-24). Calpastatin possesses four inhibitory domains which contain a consensus inhibitory sequence (TIPPEYR) which, in itself, is able to specifically inhibit calpain (17,20-24). A comprehensive analysis of how all regulatory factors interact to control calpains is needed.

Evidence that calpains mediate disassembly of the myofibrillar apparatus is compelling. Calpain initiates digestion of individual myofibrillar proteins including desmin, filamen, C-protein, tropomyosin, troponin T, troponin I, titin, nebulin, vimentin, gelsolin and vinculin (25-34). However, calpains do not degrade α-actin, α-actinin or myosin heavy chain (32,33). Calpains tend to be concentrated in the Z-disk (35), the site where disassembly begins (36). Calpains are activated by calcium, and treatment of purified myofibrils with calcium causes rapid and complete loss of the Z-disk (37). Calpains are also activated in conditions of muscle wasting. Increased calpain activity in muscle has
been reported in vitamin E deficiency (38), Duchenne dystrophy (6-9) and fasting (39). Calpain mRNA concentrations are increased markedly during fasting (40). The time course for changes in calpain activity corresponds to morphological changes in muscle (41). Based on these data, Goll et al. (36) and others (9,32-33,36,39,41,42-44) have proposed that calpain plays a significant role in myofibrillar protein degradation, especially in the disassembly of the myofibril during early stages of turnover. Degradation of the Z-disk by calpain is proposed to cause disordering of the three-dimensional architecture of the thick and thin filaments in muscle, thus making the exposed myofibril more susceptible to proteolytic attack by other, less-specific proteases (41).

Limitations to the belief that calpains cause myofibrillar proteins are that the calcium concentrations required for activity are much higher than those which are physiologically attainable. In addition, some reports have shown that the proteasome supersedes the role of calpain in myofibrillar protein degradation (2,45-49). The proteasome, a large ubiquitous ATP- and ubiquitin-dependent proteolytic system, is able to degrade actin and myosin in vitro (45). A recent study by Solomon et al. (45) indicated that the proteasome degrades intact monomeric myofibrillar proteins but not when those same proteins are associated with other myofibrillar proteins.

Limitations of previous studies are that many have been performed in vitro using non-specific protease inhibitors. To assess the function played by calpains in living muscle cells, we developed three genetic strategies to regulate calpain activity in cultured cells: over-expression of dominant negative m-calpain (DN), antisense m-calpain (AS) and calpastatin inhibitory domain (CID). Our expectation was that specific regulation of calpains in living muscle cells would allow the unequivocal identification of calpain function. Our data indicate that calpains play significant roles in L8 muscle cell protein degradation and participate in the degradation of nebulin. These data indicate that inhibition of calpains may effectively slow myofibrillar protein digestion in vivo.
Materials and Methods

Plasmid construction

*Site-directed mutagenesis of rat m-calpain:* Full length rat m-calpain cDNA, subcloned into pT7-7fn phagemid, was generously supplied by John Elce (Queens University, Canada). Site-directed mutagenesis was performed based on the classic Kunkel method and Muta-Gene T7 Enzyme Refill Pack Version 2 (Bio-Rad, Hercules, CA) with slight modification. In brief, a single-stranded DNA template was generated by super-infection with M13K07 helper phage of CJ 236 (dut ung, F') host cells containing pT7-7fn phagemid DNA. Five hours later, the culture was centrifuged at 17,000×g for 15 min at 4°C. The supernatant was collected and incubated for 10 min at 65°C to kill contaminating host cells. Centrifugation followed and 1/4 volume of 3.5 M ammonium acetate/20% PEG6000 was added to the supernatant, followed by incubation on ice water for 30 min. The precipitate was collected by centrifuging twice at 17,000×g for 15 min at 4°C. The pellet was collected and 300 μl TE was added. Purification of single-stranded DNA template was completed by phenol extraction and ethanol precipitation. The purified uracil-containing single-stranded DNA template was then stored at -20°C for further experiments. An oligonucleotide (5'-AGCCAGAAGCCAGGCGCTCCCAAGGGCTCC-3', synthesized by the Central Services Lab, Oregon State University) was phosphorylated with T4 polynucleotide Kinase (Promega, Madison, WI) as a primer for synthesis of a complementary DNA strand. The synthesis reaction of the complementary DNA strand was performed with a Muta-Gene T7 Enzyme Refill Pack (Bio-Rad). Successful mutation at 105aa (cysteine–alanine) was verified first by HgaI restriction enzyme digestion (a new restriction site generated after mutation) followed by DNA sequencing. Finally, dominant negative full-length rat m-calpain digested with Nco I and Sal I from pT7-7fn phagemid, was subcloned with Not I linkers into the pOP13CAT (Stratagene, La Jolla, CA).
expression vector following removal of the CAT gene. This plasmid was designated as pOP13DN. Correct orientation of the insert was confirmed by DNA sequencing and restriction enzyme digestion.

**Construction of calpastatin inhibitory domain:** The calpastatin inhibitory domain (CID) nucleotide sequence (Figure 2.1, Panel A) was deduced from peptide EKLGERDDTPPEYRELLERKGTV (17) with standard mammalian codon usage. A translation start codon ATG, Kozak sequence and Not I site were added sequentially to the 5'-end. A translation stop codon (TGA) and Not I site were added at the 3'-end (Figure 1, Panel B). This 97-bp full-length single-stranded CID expression construct was synthesized by the Central Services Lab. Double-stranded full length CID expression construct was generated by PCR reaction with a forward primer P1 (5'-TGCCTGCGGCATGGAGAAGCT-3') and a reverse primer P2 (5'-TGCCTGCGGCACACAGCGGT-3'). The PCR reaction was completed with 2 mM MgCl₂, 0.2 mM dNTP mix, 10 µl Taq DNA polymerase buffer, 2 µl S-S DNA template (1.3 µg/µl), 1 µl (50 pmol) primer P1 and 1 µl (50 pmol) primer P2, 2 µl Taq DNA polymerase (5 U/µl, Promega) and water under 30 cycles of 94°C for 3 min, 55°C for 2 min and 74°C for 30 sec. Double-stranded CID cDNA was then subcloned by T4 DNA Ligase (2000 U/µl, New England Biolabs, Beverly, MA) into pOP13CAT expression vector following removal of the CAT gene, and was designated pOP13CID. Correct orientation of the insertion was confirmed by DNA sequencing.

**Preparation of antisense m-calpain RNA construct:** Antisense m-calpain cDNA was generated by PCR with rat m-calpain full length cDNA as a template and a forward primer A1 (5'-TTGCGGCCGCTGATGAAA-3') and a reverse primer A2 (5'-TTGCGGCCGCTGAAAGGGAAG-3'). The PCR reaction was completed with 2 mM MgCl₂, 0.2 mM dNTP mix, 10 µl Taq DNA polymerase buffer, 80 ng of rat m-calpain cDNA, 1 µl (50 pmol) primer A1 and 1 µl (50 pmol) primer A2, 2 µl Taq DNA polymerase (5 U/µl, Promega) and water with 30 cycles of 94°C for 1.5 min, 55°C for 2 min and 74°C for 30 sec. The 165-bp PCR product was
A. CID nucleotide sequence:

gagaagctggcgagagggacgacaccatccccccgagatacagggagctgctggagaagaagaccggc
gtg

B. CID construct:

\textbf{TGC}GG\textbf{GCCGCC}AT\textit{G}gagaa--------------
gcgtg\textbf{TGA}CGGC\textbf{GCC}GCA

Figure 2.1: CID construct. A: complete CID nucleotide sequence. B. CID construct containing CID nucleotide sequence (lower case letters in the middle), Kozak sequence (bold italic), translation start codon ATG underlined, translation stop codon TGA underlined, and Not I sites (5' and 3').
verified on 2 % TAE agarose gel and subcloned in reverse orientation into the Not I site of pOP13CAT vector following removal of the CAT gene. This was designated as pOP13AS. Correct orientation of the insert was verified by restriction enzyme digestion and confirmed by DNA sequencing.

**Cell culture**

Rat L8 myoblasts and transfectants were cultured in medium containing Dulbecco's Modified Eagle Medium (DMEM, Gibco, Gaithersburg, MD) with addition of 1000 mg/l D-glucose, 584 mg/L-glutamine, 110 mg/L sodium pyruvate, 4 mg/L pyridoxine hydrochloride, 3.7 g/L sodium bicarbonate, 100 U/ml Penicillin-Streptomycin (Gibco) and 10% fetal bovine serum (characterized FBS, Hyclone, Logan, UT). When cells reached 90-95% confluence, the medium was changed to DMEM complete medium containing 2% horse serum (Hyclone) in order to obtain fully differentiated myotubes. The medium was changed every other day for either myoblast or myotubular maintenance.

For long-term storage or subculture, adherent cells were detached from the plate by trypsinization with 2.5 mg/ml trypsin and 3-min incubation at 37°C. FBS medium was immediately added to stop further trypsinization. Suspension cells were centrifuged at 1000 rpm for 5 min and the supernatant was discarded. For subculture, the cells were evenly distributed on new plates. For storage, the cells were placed in vials with a 9:1 ratio of FBS medium and DMSO (Sigma, St. Louis, MO) and stored at - 80°C or in liquid nitrogen.

**Stable co-transfection**

Calcium phosphate-based eukaryotic stable cell transfection (50) was performed in this study with these modifications. In brief, the myoblasts used for transfection were
recloned or possessed near 100% differentiation ability. On the day prior to transfection, one 10-cm plate of myoblasts at 80-90% confluence was split into four 10-cm plates. On the day of transfection, cells were fed with 9.0 ml FBS medium 4 hours prior to calcium phosphate precipitation. Plasmids to be transfected were purified by CsCl gradient centrifugation. Ten µg of each plasmid to be co-transfected was co-precipitated by ethanol, followed by a 70% ethanol wash and centrifugation. The DNA pellet was resuspended in 450 µL sterile water, and 50 µL of 2.5 M sterile CaCl$_2$ was added. The DNA/ CaCl$_2$ solution was added with a Pasteur pipet dropwise into 500 µM 2× HeBS solution containing 282 mM NaCl, 50 mM HEPES acid, and 0.8 mM Na$_2$HPO$_4$, pH 7.05. The solution was immediately vortexed for 5 sec. After 20 min at room temperature, the precipitate was distributed evenly over a 10-cm plate of cells. Each co-transfection was carried out with duplicate plates.

Twelve hours later, cells in one of each duplicate was harvested and stored in a -80°C freezer for later selection. The other plate was trypsinized and then the cells were redistributed with various dilutions. Antibiotic selection with hygromycin B (200 µg/ml, Gibco) was used for selection of cells containing the p3'SS repressor plasmid, and geneticin (400 µg/ml, Gibco) was used for selection of the cells transfected with pOP13CAT, pOP13CID, pOP13DN, or pOP13AS. Co-transfectants were cultured with FBS medium in addition to hygromycin B and geneticin.

Each clone was transferred from the original plate by a cloning ring made from the top of a 1-ml sterile blue micropipetter tip when the cell clones were large enough to be visualized without a microscope. Selected clones were cultured sequentially in a 24-well plate, a 12-well plate, a 6-well plate, a 5-cm plate and, finally, a 10-cm plate. Twenty clones of each of the co-transfectants were selected. Each clone was stored in 5 vials in a -80°C freezer. Co-transfectants were always cultured with antibiotics until the medium was changed to HS medium.

The clones co-transfected with p3'SS (repressor plasmid, Stratagene) and pOP13CAT (expression vector) were named L8/PC (plasmid control cell line). The clones cotransfected with p3'SS and pOP13DN were named L8/DN (dominant negative
cell line). The clones co-transfected with p3'SS and pOP13CID were named L8/CID (calpastatin inhibitory domain cell line). The clones co-transfected with p3'SS and pOP13AS were named L8/AS (antisense m-calpain cell lines).

**Total RNA preparation**

The cell lines L8/CID, L8/DN and L8/PC were cultured with FBS medium until they reached 90% confluence. Then, the cells were cultured for 6 hours, 12 hours and 24 hours while half of the plates were treated with 5 mM isopropyl-β-D-thiogalactoside (IPTG, Sigma). Duplicate plates were made for both treatment and control. The guanidinium method (51) for total RNA isolation was completed with slight modification. Following IPTG treatment, cells were washed with ice-cold PBS once and lysed with 500 μL (5-cm plate) denaturing solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.1 M 2-ME and 0.5% N-lauroylsarcosine. Lysate was collected with a disposable cell lifter (Fisher Scientific, Santa Clara, CA) and transferred into a 1.7-ml microcentrifuge tube. Fifty μL of 2 M sodium acetate (pH 4.0) was added and mixed thoroughly by inversion. Five hundred μL of fresh acid-saturated phenol (pH 4.3, Fisher) and 100 μL of 49:1 chloroform/isoamylalcohol were added sequentially and rigorously vortexed following in each step. The suspension was incubated 15 min on ice, followed by 20 min of centrifugation at 17,000 rpm at 4°C. Then, the upper aqueous phase was transferred to a fresh tube. The RNA was precipitated by addition of 500 μL (1 vol) of 100% ice-cold isopropanol and incubated at -20°C for at least 30 min. RNA was pelleted by centrifugation for 10 min at 17,000 rpm, at 4°C. The pellet was dissolved in 150 μl denaturing solution and precipitated by adding 150 μL of cold isopropanol with at least 30 min incubation at -20°C. The precipitate was centrifuged again at 17,000 rpm for 10 min at 4°C. The RNA pellet was washed once with 75% ethanol and incubated 15 min at room temperature to dissolve residual amounts of guanidinium contaminants. Final centrifugation was done at 17,000 rpm for 10 min at 4°C. Thirty μL of DEPC-treated H₂O
were added to each RNA pellet after drying of the pellet in a vacuum for 15 min. The RNA concentration was measured by a DNA/RNA calculator (Gene Quant II, Pharmacia Biotech, Cambridge CB4 4FJ, England). The quality of RNA was determined by running regular 1% TAE (0.04 M Tris-acetate, 0.001 M EDTA, and 1.14 mL/L glacial acetic acid) agarose gel for 6-10 min. RNA aliquots were stored in a -80°C freezer.

Reverse Transcription PCR

RT-PCR assays were performed in two separate steps: reverse transcription to generate the single-stranded cDNA, and PCR to amplify the specific cDNA. Various amounts of reverse transcription reaction were used as template in the following PCR reaction because of the variable copy number of single-stranded cDNAs generated.

Reverse transcription reaction: RNA used for the reverse transcription reaction was first treated with RQ1 DNase (RNase-free, Promega) to minimize genomic DNA contamination. The details of the reverse transcription reaction are as follows. Two µg of total RNA (plus H2O up to 9.5 µL as a final volume), 2 µL of MMLV reverse transcriptase reaction buffer (5×), 0.5 µL of RNasin (40 U/µL, Promega), and 1µL of RQ1 DNase (1 U/µL) were added sequentially and incubated one hour at 37°C. Heat inactivation was followed for 10 min at 85°C to inhibit DNase activity, and the reaction mixture was allowed to cool to room temperature. To the reaction, 6 µL of reverse transcriptase reaction buffer, 6 µL of oligo dT (18 mer, 0.25 µg/µL), 9 µL of DEPC-treated H2O, 4 µL of dNTP (10 mM each in 10 mM Tris-HCL, pH 7.5), 2 µL of BSA (2 µg/µL) and 1 µL of M-MLV reverse transcriptase (200U/µL, Promega) were added sequentially and incubated 2 hours at 37°C. At the end of the reaction, 0.5 µL of RNase ONE (5 U/µL, Promega) were added and the reaction was continued for another 15 min at 37°C to digest RNA. This was followed by heat inactivation of RNase and transcriptase for 5 min at 95°C.
**PCR reaction:** The m-calpain RT-PCR reaction consisted of 2 µL of reverse transcription (RT) reaction as a template, 1.5 mM MgCl₂, 0.2 mM dNTP, 1× Taq DNA polymerase buffer, 0.5 µL of Taq DNA polymerase (Promega), 1 µL of forward primer RTantiP (5'-GATGAAACTGGCACAAGA), 1 µL of reverse primer m-P2 (5'-AGCTCCTGACGGACCTAGATG-3'). The reaction was performed for 30 cycles at 94°C for 1 min, 55°C for 1.5 min, and 74°C for 1 min. pOP13DN was used as the template in one reaction as a positive control of the PCR reaction.

The CID (calpastatin inhibitory domain) RT-PCR reaction consisted of 10 µL of RT reaction as a template, 1.5 mM MgCl₂, 0.2 mM dNTP, 1× Taq DNA polymerase buffer, 0.5 µL of Taq DNA polymerase, 1 µL of forward primer P1 (5'-CATGGAGAAGCTGGGCGA-3'), and 1 µL of reverse primer P2 (5'-TCACACGACGGTTCTTCTT-3'). The reaction was performed for 30 cycles at 94°C for 1 min, 55°C for 1.5 min, and 74°C for 30 s. pOP13CID was used as the template in one reaction as a positive control.

The chloramphenicol acetyl transferase (CAT) RT-PCR reaction (positive control for IPTG induction) consisted of 10 µL of RT reaction as a template, 3 mM MgCl₂, 0.2 mM dNTP, 1× Taq DNA polymerase buffer, 0.5 µL of Taq DNA polymerase, 1 µL of forward primer CAT1 (5'-ATGGAGAAGCTGGGCGA-3'), 1 µL of reverse primer CAT2 (5'-TTACGCCCCGCTGCTCCGACTCAT-3'). The reaction was performed for 30 cycles at 94°C for 1 min, 55°C for 1.5 min, and for 74°C for 1 min. pOP13CAT was used as a template in one reaction as a positive control.

The LacI RT-PCR reaction (negative control for IPTG induction) consisted of 6 µL of RT reaction as a template, 1.5 mM MgCl₂, 0.2 mM dNTP, 1× Taq DNA polymerase buffer, 0.5 µL of Taq DNA polymerase, 1 µL of forward primer lacP1 (5'-TGTCGATGGTAGAAGGAAG-3'), and 1 µL of reverse primer lacP2 (5'-GTGGTGGTTTCTTTTCCACAG-3'). The reaction was performed for 30 cycles at 94°C for 1 min, 54°C for 1.5 min, and 74°C for 1 min. p3'SS was used as the template in one reaction as a positive control for the PCR reaction. All of the primer solutions contained 50 pmol/µL.
Fifteen µL of each RT-PCR reaction were used for gel electrophoresis using 1% TAE agarose gel for the 1100-bp m-calpain RT-PCR product, the 680-bp CAT gene RT-PCR product and the 600-bp lacI RT-PCR product. A 2% TAE agarose gel was used for the 80-bp CID RT-PCR product.

**Measurement of total protein degradation**

After 6 days of myotubular differentiation, all of the plates were supplied with 2 µCi/ml of ³H-tyrosine (NET-127, DuPont NEN, Boston, MA) and 5 mM IPTG was added to one-half of the plates. Twenty-four hours later, the plates was washed twice with DMEM medium containing additional 2 mM cold tyrosine (chase) and refilled with DMEM medium or HS medium. At this time, 1.5 ml of medium were taken from each plate and radioactivity was measured by scintillation counter. This was designated as the radioactivity present at time zero. Time zero plates from cultures not treated with IPTG were described as T₀ whereas time zero plates from cultures treated with IPTG were described as T₀t. The rest of the plates were cultured for an additional 6 or 12 hours. Among those, half continued to be treated with 5 mM IPTG. Six hours (T₆ or T₆t) or 12 hours (T₁₂ or T₁₂t) later, 1.5 ml of medium from each plate was taken and radioactivity was measured. From these measurements, total protein degradation at 6 hours (D₆ or D₆t) or 12 hours (D₁₂ or D₁₂t) was determined as follows: 

\[ D₆ = T₆ - T₀ \]
\[ D₁₂ = T₁₂ - T₀ \]
\[ D₆t = T₆t - T₀t \]
\[ D₁₂t = T₁₂t - T₀t \]

The percent reduction of total protein degradation (R) caused by addition of IPTG was calculated as follows:

\[ R₆ = (D₆ - D₆t) / D₆ \times 100 \]
\[ R₁₂ = (D₁₂ - D₁₂t) / D₁₂ \times 100 \]
Protein extraction from cell culture

The methods used in this study for recovery of cell protein were similar to those reported by Wang et al (52). Following treatment, medium was removed and attached cells were washed twice with PBS-EDTA (phosphate-buffered saline plus 100 mM EDTA). Cells were then lysed at room temperature for 10-15 min with the addition of a 500 µl (for 5-cm plate) lysis buffer containing 2% (w/v) SDS, 5 mM EGTA, 5 mM EDTA, 0.5 mM PMSF (phenylmethysulfonyl fluoride), 10 µg/ml AEBSF (4-(2-aminoethyl)-benzenesulfonylfluoride), 5 µg/ml TLCK (N-α-p-tosyl-L-lysine chloromethyl ketone), 0.01% (w/v) leupeptin, 10 µg/ml TPCK (N-α-p-tosyl-L-phenylalanine chloromethyl ketone), and 20 mM Tris-HCl (pH 7.4). One hundred µl of 100% (w/v) trichloroacetic acid (TCA) was added to the lysate. The DNA aggregate was then removed. After centrifugation (3600×g for 5 min), pellets were washed with 1 ml of 2.5% (w/v) TCA. The final pellets were neutralized with 40-80 µl of 3 M Tris base. The protein concentration was measured by using a protein assay solution (BioRad).

Electrophoresis and Immunoblotting

Thirty to 50 µg of protein samples were run on SDS-PAGE (60V, 4 h) in a PROTEAN II xi Vertical Electrophoresis unit (Bio-Rad) with Tris-glycine running buffer system (25 mM Tris base, 192 mM glycine, 0.1% (w/v) SDS (pH 8.3) and transferred onto an OPTITRAN membrane (supported nitrocellulose, Schleicher & Schuell, Keene, NH) at 0.11 A overnight in a Tris-glycine buffer system with addition of 20 % methanol and without SDS using a Trans-Blot Cell unit (Bio-Rad). A Bio-Rad high molecular weight marker was used as a standard. A 4%-12.5% gradient gel with a 100:1 ratio of acrymide to bis and no stacking gel was used for nebulin protein separation. A Tris-glycine transfer buffer system with addition of 0.1% SDS and 15% methanol was used for nebulin transfer at 90 V for 150 min. A 7.5% separating gel and 5% stacking gel were
used for m-calpain and lacI protein separations. A 4-20 % gradient gel and 4 % stacking gel were used for fodrin protein separation.

The blots were rinsed with TBS (50 mM Tris-base and 150 mM NaCl), blocked by 5 % skim milk in TTBS (TBS plus 0.05% Tween-20) for one hour and probed with nebulin (1:5000, A-9891, Sigma), lacI (1:1000, Stratagene), m-calpain (1:2000), and spectrin antibodies (1:1000, Chemicon, Temecula, CA) for 2.5 hours. After a 30 min wash with TTBS following antibody probing, the blots were transferred into a secondary antibody solution with 1:5000 dilution of either anti-rabbit or anti-mouse IgG (BioRad) conjugate for at least 30 min. After another 30-min wash, the blots were developed with either an ECL (Amersham, Arlington Heights, IL) or a LumiGLO (KPL, Gaithersburg, MD) detection system. Blots that have been shown here represent one of the at least three similar results. Blots (Fig. 2.6 (E)) were quantified by scanning densitometry. The Object Average method of Image QuaNT, Molecular Dynamics software, was used to determine the amount of each immune image based on the area and intensity of the blot. To allow comparison of results of different experiments, each membrane was reprobed with an internal standard, m-calpain, and results were compensated for variations in these values.

Production of m-calpain antibody

An antipeptidic antibody against the amino-terminal 18-mer segment of pre-autolysis m-calpain was developed using a synthetic peptide (53), AGIAAKLAKDREAEGLC, conjugated to keyhole limpet hemocyanin and affinity-purified with Imject Activated Immunogen conjugation Kits (Pierce, Rockford, IL). A female New Zealand white rabbit, 12-week old, was injected with the immunogen. Antiserum m-calpain was collected in 4 weeks after injection and was purified with Sulfolink coupling gel (Pierce, Rockford, IL).
Statistical analysis

Raw data were analyzed using a two-way ANOVA table within 6 hour and 12 hour groups. The F-test in the ANOVA table was used to evaluate any significant differences amongst the means. Multiple Range Tests indicate the significant difference between each others.
Results

Following transfection, cells were selected for resistance to both hygromycin B and geneticin. Ability to differentiate was tested for each transfectant. Only the transfectants which were able to differentiate fully were used for further experiments.

Over-expression of the dominant negative m-calpain and calpastatin inhibitory domain; detection at the mRNA level

To verify that cell lines expressed constructs as desired, several confirmation studies were completed. In the first of these (Figure 2.2), we assessed effects of IPTG treatment (12 and 24 hours) on CAT, m-calpain and Lac I mRNAs by RT-PCR in the L8/PC cell line. IPTG induced expression of CAT without affecting m-calpain or LacI expression (see Panel A). In the L8/DN cell line, addition of IPTG for 12 or 24 hours increased expression of m-calpain mRNA but did not affect LacI (Figure 2.2; Panel B). Of interest, the signal for m-calpain shown in Panel B represents both the endogenous and dominant negative m-calpain and our expectation is that the increase in signal is due to synthesis of dominant negative m-calpain. In the third study (Figure 2.2; Panel C), we assessed effects of IPTG on CID mRNA, m-calpain mRNA and LacI mRNA. IPTG caused a large increase in CID mRNA but did not affect m-calpain and LacI mRNAs. The signal for CAT (Panel A) and CID mRNA (Panel C) in the absence of IPTG indicates the “leakiness” of the LacSwitch expression system. These results demonstrate that exogenous gene mRNA was inducible by IPTG and stable for at least 24 hours.
Figure 2.2 (A): IPTG induction of exogenous gene expression in L8/PC cell line by RT-PCR assay. All of the transfectants were grown to 90-100% confluence, after which IPTG (5 mM) was added to half of the plates and incubated for 12 or 24 hours. RT-PCR was performed as described in text.
Figure 2.2 (B): IPTG induction of exogenous gene expression in L8/DN cell line by RT-PCR assay. All of the transfectants were grown to 90-100% confluence, after which IPTG (5 mM) was added to half of the plates and incubated for 12 or 24 hours. RT-PCR was performed as described in text.
Figure 2.2 (C): IPTG induction of exogenous gene expression in L8/CID cell line by RT-PCR assay. All of the transfectants were grown to 90-100% confluence, after which IPTG (5 mM) was added to half of the plates and incubated for 12 or 24 hours. RT-PCR was performed as described in text.
Effects of over-expressing dominant negative and antisense m-calpain RNA on m-calpain at the protein level.

To address the question of whether dominant negative m-calpain would be expressed at the protein level and whether over-expression of antisense mRNA would reduce endogenous m-calpain protein concentration, Western blot analysis was performed (Figure 2.3). In L8/PC and L8/CID cell lines (Panels A and B), addition of IPTG did not affect m-calpain and LacI concentrations as expected. However, in the L8/DN cell line (Panel C), total m-calpain protein was increased following addition of IPTG at 8 hours. In the L8/AS cell line, reduction of endogenous m-calpain protein was almost complete following IPTG supplementation for 8 hours. Following this, m-calpain concentration increased gradually. These results demonstrate that m-calpain antisense RNA blocks m-calpain protein expression successfully within an 8-hour period, and dominant negative m-calpain was over-expressed at the protein level in transfectants.

Effects of over-expressing of CID, dominant negative m-calpain and antisense m-RNA on fodrin degradation

Fodrin, a nonerythroid spectrin and a multifunctional protein, is a major component of the cortical cytoskeleton of most eukaryotic cells (54-55). Fodrin possesses binding sites for many proteins, including actin, calmodulin, and CD5 (54). Proteolysis of fodrin by calpain has been observed during several processes. Calpains cleaved fodrin into 150 and 145 kDa fragments following addition of A23187 (Ca$^{++}$ ionophore) and maitotoxin, which stimulates calcium influx in both excitable and nonexcitable cells, in SH-SY5Y neuroblastoma cells and cerebrocortical cultures (52,56). Because fodrin is a well-characterized calpain substrate, we used fodrin degradation as an index of calpain activity in rat L8 myotubes.
Figure 2.3: Effect of over-expression of various constructs on calpain concentration in transfectants. Myoblasts were cultured to 90-100% confluence, after which IPTG (5 mM) was added to each plate. All the plates but '0' were cultured for an additional 8 h, 1 day, 2 days, 3 days, 4 days, 5 days. Western blots were performed and polyclonal rat m-calpain antibody and polyclonal Lac I antibody were used to probe the blots as described in the text. Panel A: L8/PC cell line; Panel B: L8/CID cell line; Panel C: L8/DN cell line; Panel D: L8/AS cell line.
Figure 2.4: Inhibition of fodrin breakdown product by reduction of calpain activity in various cell lines. Panel A; Fodrin degradation in L8/PC cell line, cells were cultured with no A23187 treatment, A23187 (10 µM) for 1 hour, A23187 (20 µM) for 2 hours, respectively. Panel B represents fodrin degradation in the presence and absence of IPTG in the cell lines L8/CID, L8/DN and L8/AS exposed to A23187 (20 µM) for 2 hours. Monoclonal spectrin antibody (non-erythroid) was used for Western blotting.
To study the impact of over-expression of CID, DN-m-calpain and m-calpain antisense on endogenous calpain activity, we examined fodrin breakdown following addition of A23187 to culture. In L8/PC cells, fodrin was cleaved into 145/150 kDa fragments following addition of 10 µM or 20 µM A23187 within a one-hour incubation period (Figure 2.4, Panel A). In L8/CID and L8/DN cell lines, fodrin breakdown following addition of 20 µM A23187 within 2 hour incubation was reduced by addition of IPTG (lanes b and d; Panel B, Figure 2.4). These results demonstrate that endogenous calpain activity is reduced by over-expression of dominant negative m-calpain or calpastatin inhibitory domain.

**Contribution of calpain to total protein degradation**

Total myofibrillar protein degradation following serum withdrawal was measured with a ³H-tyrosine labeling experiment. Figure 2.5, Panel A shows that IPTG did not affect total protein degradation following serum withdrawal in the L8/PC cell line. However, in the L8/DN cell line (Figure 2.5, Panel B), total protein degradation was reduced by 30 % by over-expression of the dominant negative m-calpain for 12 hours. In the L8/CID cell line (Figure 2.5, Panel C) total protein degradation was reduced by 63 % following over-expression of calpastatin inhibitory domain for 12 hours. IPTG also caused a 33% reduction in degradation at 6 hours in the L8/CID cell line. These data demonstrate that a large portion of myotube protein degradation is mediated by calpains.

**Stabilization of nebulin by calpains**

Nebulins, a family of giant proteins with molecular masses of 600 to 900 kDa in various skeletal muscle tissues (53,57), are the sole constituent of a set of long inextensible longitudinal filaments that span the space between the Z-disk and the distal
Figure 2.5 (A): No effect of IPTG on total protein degradation in L8/PC cell line. Degradation was assessed for 6 hours and 12 hours. Values represent the mean of three repeats. Triplicates were done for each experiment.
Figure 2.5 (B): Statistical significant effect of IPTG on total protein degradation at 12 hours following serum deprivation in L8/DN cell line. Degradation was assessed for 6 hours and 12 hours following serum deprivation. Values represent the mean of three repeats. Triplicates were done in each experiment.
Figure 2.5 (C): Statistical significant effect of IPTG on total protein degradation at 6 hours and 12 hours following serum deprivation in L8/CID cell line. Degradation was assessed for 6 hours and 12 hours following serum deprivation. Values represent the mean of three repeats. Triplicates were done in each experiment.
region of a thin (actin) filament (58). An entire nebulin filament consists of a combination of 200-, 180-, 40, 35- and 23- kDa sub-fragments (59). In order to determine whether the calpains degrade this important structural protein, we assessed nebulin degradation in our cell lines.

Fully differentiated transfectants, L8/PC, L8/CID, L8/DN and L8/AS cell lines, were divided into two groups. One group was exposed to IPTG for 24 hours (L8/PC, L8/CID, and L8/DN cell lines). The other group (L8/AS cell line) was exposed to IPTG for 8 hours. At the end of treatment, all the cells were transferred to serum-free medium and incubated for 12 more hours in the presence (Figure 2.6, lanes c and d) or absence (lanes a and b) of IPTG. Western blots were performed and monoclonal nebulin antibody was utilized as a probe (Figure 2.6; Panel A). Scanning densitometry results are shown in Panel B. It did not have any impact on the 200-kDa degradation product of nebulin in L8/PC cells (Panel A). Nebulin was stabilized by 20% following over-expression of DN-m-calpain, 25% following over-expression of antisense m-calpain, and 33% following over-expression of CID. These results indicate the involvement of calpain system in degradation of a key myofibrillar protein in living cells.
Figure 2.6: Stabilization of nebulin by reduction of calpain activity. The four cell lines (L8/PC, L8/DN, L8/AS and L8/CID) were exposed to IPTG (5 mM) for either 24 hours (PC, DN and CID) or 8 hours (AS), after which nebulin concentration was assessed by Western blotting (Panels A-D).
Figure 2.6 (E): Representing scanning densitometry of data shown in Panels A though D. Nebulin was stabilized by 20% following over-expression of DN-m-calpain, 25% following over-expression of antisense m-calpain, and 33% following over-expression of CID.
Discussion

Accumulation and maintenance of muscle mass is dependent on control of both protein synthesis and protein degradation. In recent years, interest has focused on degradation because this process is typically accelerated in a wide variety of pathologic conditions. For example, degradation is enhanced in cachexia (2), AIDS wasting (4), various endocrine disorders (5), genetic muscular dystrophies (6-9), and in extended bed rest (3). Degradation may also be viewed as a constraint to growth (60).

The protease(s) which mediates degradation needs to be identified as this would provide a logical target for intervention. In this regard, two proteolytic systems have been studied; the proteasome and calpain. The proteasome is a large multi-functional enzyme complex which requires ATP for its activity. It is ubiquitously expressed and believed to degrade a wide range of substrates (22). Several recent studies point to the involvement of the proteasome in myofibrillar protein degradation (2,45-49); however, it is not clear whether proteasome is the key rate-limiting protease or is simply one protease in the pathway of degradation. A recent study by Solomon et al (45) indicated that the proteasome can degrade monomeric (free) myofibrillar proteins but not the same proteins when associated with other sarcomeric proteins. These data suggest that another protease must exist upstream of the proteasome to provide it with monomeric myofibrillar proteins as substrate.

Several lines of evidence suggest that calpains may be the rate-limiting protease; however, this has not been established in living cells. In the test tube, calpains degrade many myofibrillar proteins (e.g. desmin, filamin, C-protein, tropomyosin, troponin T, troponin I, titin, nebulin, vimentin, gelsolin, and vinculin; 25-34). Changes in activities also correlate with rates of degradation. Despite this, questions regarding calpain participation in this process have been raised. For example, high concentrations of Ca++ are required for activity, at least in vitro, and it is not clear how calpains could ever be activated in the low Ca++ micro-environment of the cell. Thus, the overall goal of this work was to define more
precisely the role of calpains in protein degradation. Three indices of degradation were assessed: fodrin degradation, total protein degradation and nebulin degradation.

We utilized cell culture as our study model and three genetic strategies to manipulate endogenous calpain activities: over-expression of dominant negative m-calpain, over-expression of calpastatin inhibitory domain, and over-expression of antisense m-calpain mRNA. We proposed that over-expression of any of these constructs would inhibit endogenous calpain activities. Either over-expression of dominant negative m-calpain and antisense m-calpain mRNA was expected to specifically inhibit m-calpain activity. Over-expression of calpastatin inhibitory domain would inhibit both μ- and m-calpain activities because calpastatin has been shown to inhibit both calpain isoforms (17,20-24). The ability of these strategies to affect the calpain system was assayed by: 1) calpain mRNA concentrations were assessed by RT-PCR; 2) m-calpain concentrations were assessed by Western blot; 3) endogenous calpain activity was evaluated by monitoring breakdown of fodrin. For analysis of calpain function in myofibrillar protein breakdown, we measured total protein degradation and tested the stability of nebulin, an α-actin- and Z-disk-binding protein. Our results showed that exogenous genes (dominant negative m-calpain, calpastatin inhibitory domain, and antisense m-calpain) were over-expressed in an inducible manner at the mRNA level (Figure 2.2, Panels B and C) and/or at the protein level (Figure 2.3, Panels C and D). Endogenous calpain activity was reduced by over-expression of dominant negative m-calpain and calpastatin inhibitory domain (Figure 2.4, Panel B).

This work first established that expression of the various constructs regulated the calpain system as expected. To accomplish this, we evaluated fodrin degradation. Fodrin is a well-charactered calpain substrate (52,56,61,62). In fact, specific calpain cleavage sites of fodrin have been identified (63). Our data clearly show that over-expression of our various constructs (Figure 2.4, Panel B) stabilized fodrin. Hence, these data provide evidence for our ability to control calpain activity in living muscle cells.

The data above also showed that calpains were involved in total protein degradation. This was evaluated by release of radiolabeled tyrosine into cell culture.
medium from prelabeled protein. Over-expression of CID in L8/CID cells caused a 63% reduction in total protein degradation whereas over-expression of dominant negative m-calpain in L8/DN cells caused only a 30% reduction. Our expectation was that the CID construct should inhibit both \( \mu \)- and m-calpain whereas the DN-m-calpain construct should specifically inhibit m-calpain by competing with endogenous intact m-calpain for substrate. If so, the difference in inhibition caused by these two constructs may approximate the degradation in L8 cells which is mediated by other calpains (i.e., \( \mu \)-calpain). A more direct means of assessing the role of \( \mu \)-calpain in muscle cells would be to over-express a dominant negative \( \mu \)-calpain construct, similar to that used in this study. This work is in progress.

Many people believe that calpains are rate-limiting enzymes in myofibrillar protein degradation (41-43). Yet, other compelling data suggest that the proteasome also participates in this process (2,45-49). A recent article by Solomon et al. (45) indicated that activity of the proteasome could be secondary to another protease because it was capable of degrading monomeric myofibrillar proteins, not higher structures. We chose to investigate the possibility that calpain degrades myofibrillar protein by investigating nebulin. While intact nebulin is near 600 kDa, it undergoes autofragmentation into 200-, 180-, 40-, 35- and 23- kDa sub-fragments in the presence of 0.1 mM Ca\(^{++}\) (59,60). The 200 kDa fragment was the principle nebulin fragment recovered from muscle preparations in our experiment. We found that inhibition of calpain activity, whether by over-expression of CID, AS or DN, stabilized the 200-kDa fragment of nebulin. These data indicate that calpain degrades nebulin and therefore, plays an important role in myofibrillar protein degradation.

One of our cell lines allowed for over-expression of antisense m-calpain mRNA. We found that production of endogenous m-calpain was almost completely blocked by over-expression of antisense m-calpain mRNA 8 hours after induced by 5 mM IPTG. This result differs from those of Zhang et al. (64), who reported that m-calpain was a long-lived protein. Rapid reduction in m-calpain content indicates that m-calpain turns over in muscle cells with a half-life of less than 8 hours. The reason Zhang et al. (64) detected a
half life longer than this could be due to instability of antisense oligomers used in their study. Also of interest in our study was the ability of L8/AS muscle cells to increase m-calpain concentrations between 8 and 24 hours of antisense induction. These data imply that L8 cells are able to "sense" a reduced m-calpain concentration and compensate by increasing m-calpain synthesis. Maintenance of stable protease concentrations in cells is needed for normal homeostasis. The transient down-regulation of calpain activity by antisense may account for the inability of this strategy to measurably affect fodrin degradation (Figure 2.4, Panel B). Our earlier results have shown that calpain concentrations remain unchanged despite large changes in muscle cell physiology (e.g. fasting) and that stable concentrations of calpain are maintained by dramatic changes in calpain mRNA (40). The observations here are consistent with the belief that muscle cells regulate protease concentrations precisely.

Earlier studies with L8 cells (65) have documented that serum withdrawal caused an approximate 20% increase in total protein degradation. It is interesting that stabilization of nebulin and of total proteins by over-expression of the calpain system was detectable only under conditions of serum starvation (i.e. accelerated degradation). These data indicate that calpain may function in degrading proteins under stimulated conditions but may not account for the normal turnover of proteins. Alternatively, the condition of accelerated degradation which is caused by serum withdrawal may have created a larger "target" for regulation and, in this context, ability to detect a role for calpain may be improved by this strategy.

In conclusion, calpains play definitive roles in protein breakdown in muscle cells. They degrade fodrin, a well-characterized calpain substrate in vivo and account for a large proportion of total protein degradation. m-Calpain accounts for roughly 50% of the degradation of protein mediated by all calpains. Calpains degrade nebulin, an important architectural protein of the sarcomere and may be particularly important to degradation during pathological conditions. In addition to these roles, we also discovered that m-calpain is a short-lived protein in muscle cells and that L8 cells have the ability to quickly
adapt to an m-calpain "deficiency". Studies are under way to discover other substrates for calpains in muscle and to elucidate roles of μ-calpain as well.

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References


Chapter III

Regulation of Poly(ADP-ribose) Polymerase by Calpain in Rat L8 Myoblasts

Jing Huang and Neil E. Forsberg

To submit to BBA
Abstract

In many degradative and metabolic muscle diseases cells die without a marked inflammatory response. A potential mechanism for accomplishing this is apoptotic cell death. Involvement of calpain in apoptosis has been observed. More recently, calpain-dependent degradation of poly(ADP-ribose) polymerase (PARP) in vitro was reported. PARP cleavage is a hallmark of apoptosis in many types of cells. The goal of this work was to investigate whether calpain mediates PARP degradation in myoblasts and in the presence of apoptosis inducers, A23187 and etoposide. We have established a genetic approach to inhibit endogenous calpain activity by over-expressing calpastatin inhibitory domain (CID), a calpain endogenous inhibitor. We observed that inhibition of calpain activity (indicated by over-expressing CID) (1) increased PARP concentration when post-confluent myoblasts were induced to differentiate; (2) prevented PARP degradation induced by A23187; and (3) prevented PARP degradation induced by etoposide. These data demonstrate that calpain is involved in regulation of PARP concentration in differentiating myoblasts, indicating the possibility that calpain plays a role in apoptosis of differentiating myoblasts.

Key words: PARP, calpain, apoptosis, calpastatin.
Introduction

Poly(ADP-ribose) polymerase (PARP, EC 2.4.2.30) is bound to chromatin but can be solubilized with high salt and purified to homogeneity (1). The enzyme protein is composed of three domains: DNA-binding, automodification and NAD-binding domains. DNA-strand breaks are essential for PARP activation (2,3). During DNA repair, PARP, activated by DNA-strand breaks, catalyzes the transfer of an ADP-ribose moiety from its substrate, NAD, to a limited number of nuclear protein receptors involved in either chromatin architecture or DNA metabolism (4), such as histone H1, topoisomerase I, calcium-magnesium-dependent endonuclease and DNA polymerase β (5). Poly(ADP-ribosylation) of nuclear proteins is suggested to facilitate the activity of some DNA repair enzymes or relaxation of the DNA helix so that the DNA repair enzymes could access the DNA breaks (6). However, ADP-ribosylation of these enzymes in intact cells has not been demonstrated (1). Poly(ADP-ribose) synthesis has been suggested to be involved in various physiological events, including DNA excision repairs, DNA recombination, DNA replication, cell differentiation, cell growth and transformation (1). More recently, studies in a variety of cell types undergoing spontaneous or induced apoptosis have demonstrated that the 116-kDa PARP molecule is cleaved to 36-kDa and 85-kDa fragments, which separate its DNA-binding domain (36 kDa) from its NAD-binding catalytic domain (85 kDa), before or concomitant with degradation of nuclear DNA into nucleosomal fragments (7-9). Cleaved PARP is a hallmark of apoptosis in many mammalian cells (10,11), but not all (12,13). Interestingly, the observation that PARP knockout mice have an apparently normal phenotype except for skin hyperplasia (12) raised questions about the role of this enzyme in normal cellular physiology.

Calpains are calcium-activated cysteine proteases which were originally identified in porcine muscle (14,15). Two ubiquitous isoforms are well-characterized (μ- and m-calpain) and several tissue-specific isoforms have also been reported (16). Calpain substrates (16) include cytoskeletal proteins; actin-binding proteins (fodrin, spectrin, talin, filamin); microtubule-associated proteins; membrane proteins such as growth factor
receptors; enzymes such as kinases, phosphatases and phospholipases; cytokines (e.g. interleukin 12); transcription factors (Fos, Jun); tumor suppressor p53 (17,18); PARP (19); transforming growth factor-β (TGF-β) (20). Calpains have been suggested to be involved in various physiological and pathological events, including myoblast differentiation (21), muscle protein degradation (22-27), platelet activation (28-30), muscular dystrophy- or atrophy-associated diseases (31,32), and brain-associated disease (17) such as ischaemia and Alzheimer’s disease. More recently, several lines of evidence suggest the involvement of calpain in apoptosis.

Evidence that calpain is involved in apoptosis is compelling. In thymocytes, calpain inhibitors PD150606, E-64d, MDL28170 and calpain inhibitor 1, prevent dexamethasone-induced apoptosis, but not valinomycin- or heat-shock-mediated apoptosis (33). In primary cultures of hepatocytes, calpain inhibitors (I and II) attenuated TGF-β-induced apoptosis (34). Calpain inhibitors also blocked apoptosis in murine T cell lymphocytes triggered by engagement of the T cell receptor, but not apoptosis induced by steroids (35). Calpain inhibitors blocked activation-induced apoptosis of HIV-infected T lymphocytes and restored effective immune response in these cells in vitro (36). In addition, influx of calcium, causing activation of calpain, has been observed in many types of apoptosis. Calcium ionophores, A23187 (37,38) and ionomycin (39,40), induce apoptosis, while calcium channel blocker block the apoptosis process (41,42). Finally, more recently, degradation of PARP by calpain has been demonstrated in vitro (19). However, it has not be demonstrated that calpain degrades PARP in living cells. Therefore, the goal of this study is to determine whether calpain degrades PARP in L8 cultured cells.

Apoptosis is an active form of cellular death, or suicide, which plays an important physiologic role during organ development and in cellular turnover in differentiated tissues. Apoptosis has also been demonstrated to occur in response to hypoxic/ischemic (43), oxidative (44), or drug-induced (45) injuries and is thus involved in disease pathogenesis. In muscle, apoptosis has been demonstrated in differentiated myocardial muscle (46), neonatal skeletal muscle (43), skeletal myoblasts in response to injury (47),
and wound-healing muscle (48). In cell culture studies, apoptosis occurs in differentiated murine C2 skeletal muscle cells that have been injured by anabolic steroids (47) and induced by etoposide and puromycin (49), and in mouse C2 C12 cells induced by lack of merosin, a predominant laminin variant in skeletal muscle basement membranes (50). However, previous studies have shown that the apoptosis pathway and regulators of the process vary with different types of cells. Whether apoptosis takes place in skeletal muscle L8 cells is still uncertain. Given that PARP is a hallmark of apoptosis, can apoptosis inducers A23187 and etoposide induce PARP degradation in L8 cells? Does calpain participate in the mediation of PARP concentration under these conditions?

To answer those questions and to overcome non-specificity of calpain inhibitors which have been used in many studies, we have established a genetic approach to inhibit endogenous calpain activity by over-expressing the inhibitory domain of calpastatin (endogenous calpain inhibitor), which is responsible for specific inhibition of calpain (51-54). We found that inhibition of calpain, through over-expression of calpastatin inhibitory domain (CID), (1) increased intact PARP concentration in L8 myoblasts; and (2) stabilized PARP upon addition of etoposide or A23187. Our data indicate the involvement of calpain in modulation of PARP concentration in L8 cells.
Materials and Methods

Construction of calpastatin inhibitory domain

Construction of calpastatin inhibitory domain was described in detail in Chapter II. Briefly, the calpastatin inhibitory domain (CID) nucleotide sequence was deduced from peptide EKLGERDDTIPPEYRELLEKKTGV (51-54) with standard mammalian codon usage. A translation start codon ATG, Kozak sequence and NotI site were added sequentially to the 5'-end. A translation stop codon (TGA) and NotI site were added at the 3'-end. This 97-bp full-length single-stranded CID expression construct was synthesized by the Central Services Lab at OSU. Double-stranded full length CID expression construct was generated by PCR reaction with a forward primer P1 (5'-TGCGGCGCTGGAGAAGCT-3') and a reverse primer P2 (5'-TGCGGCGCTCACACGCGGT-3'). Double-stranded CID cDNA was subcloned into pOP13CAT expression vector and designated pOP13CID. Correct orientation of the insertion was confirmed by DNA sequencing.

Cell culture

Rat L8 myoblasts and transfectants were cultured in medium containing Dulbecco's Modified Eagle Medium (DMEM, Gibco, Gaithersburg, MD) with addition of 1000 mg/l D-glucose, 584 mg/L-glutamine, 110 mg/L sodium pyruvate, 4 mg/L pyridoxine hydrochloride, 3.7 g/L sodium bicarbonate, 100 U/ml penicillin-streptomycin (Gibco) and 10% fetal bovine serum (characterized FBS, Hyclone, Logan, UT). The medium was changed every other day for either myoblast maintenance.

For long-term storage or sub-culture, adherent cells were detached from the plate by trypsinization with 2.5 mg/ml trypsin and 3-min incubation at 37°C. Fetal bovine serum
medium was immediately added to stop further trypsinization. Suspension cells were centrifuged at 1000 rpm for 5 min and the supernatant was discarded. For sub-culture, the cells were evenly distributed on new plates. For storage, the cells were placed in vials with a 9:1 ratio of FBS medium and DMSO (Sigma, St. Louis, MO) and stored at -80°C or in liquid nitrogen.

Stable co-transfection

Calcium phosphate-based eukaryotic stable cell transfection (55) was performed in this study with these modifications. The method was described in detail in Chapter II. In brief, myoblasts used for transfection were recloned or possessed near 100% differentiation ability. Ten µg of each plasmid to be co-transfected were used in each transfection. Duplicate plates of each co-transfection were completed. Twelve hours later, antibiotic selection with hygromycin B (200 µg/ml, Gibco) was used for selection of cells containing the p3'SS repressor plasmid, and geneticin (400 µg/ml, Gibco) was used for selection of the cells transfected with pOP13CAT or pOP13CID.

The clones co-transfected with p3'SS (repressor plasmid, Stratagene) and pOP13CAT (expression vector) were named L8/PC (plasmid control cell line). The clones co-transfected with p3'SS and pOP13CID were named L8/CID (calpastatin inhibitory domain cell line).

Protein extraction from cell culture

The methods used in this study for recovery of cell protein were similar to those reported by Wang et al (59) and was described in detail in Chapter II. Following treatment, medium was removed and attached cells were washed twice with PBS-EDTA (phosphate-buffered saline plus 100 mM EDTA). Cells were then lysed at room
temperature for 10-15 min with the addition of a 500 µl (for 5-cm plate) lysis buffer (see Chapter II). The final pellets were neutralized with 40-80 µl of 3 M Tris base. The protein concentration was measured using a protein assay solution (BioRad).

Electrophoresis and Immunoblotting

Fifty µg of protein samples were run on SDS-PAGE (60V, 4 h) in a PROTEAN II xi Vertical Electrophoresis unit (Bio-Rad) with Tris-glycine running buffer system (25 mM Tris base, 192 mM glycine, 0.1% (w/v) SDS (pH 8.3) and transferred onto an OPTITRAN membrane (supported nitrocellulose, Schleicher & Schuell, Keene, NH) at 0.11 A overnight in a Tris-glycine buffer system with addition of 20 % methanol and without SDS using a Trans-Blot Cell unit (Bio-Rad, Hercules, CA). A Bio-Rad high molecular weight marker was used as a standard. A 7.5% separating gel and 5% stacking gel were used for PARP protein separations.

The blots were rinsed with TBS (50 mM Tris-base and 150 mM NaCl), blocked by 5 % skim milk in TTBS (TBS plus 0.05% Tween-20) for one hour and probed with anti-PARP mouse monoclonal antibody (clone:C2-10, 1:500, Biomol, Plymouth Meeting, PA) for 2.5 hours. After a 30 min wash with TTBS following antibody probing, the blots were transferred into a secondary antibody solution with 1:5000 dilution of anti-mouse IgG (BioRad) conjugate for at least 30 min. After another 30 min wash, the blots were developed with either an ECL (Amersham, Arlington Heights, IL) or a LumiGLO (KPL, Gaithersburg, MD) detection system. All the blots present in this paper represent one of the at least three similar results. Quantity of each blot determined by scanning densitometry was described in Chapter II.
Results

We have observed routinely that induction of myoblast differentiation, especially post-confluent myoblasts, causes some cells to detach and die. It has been reported that differentiated apoptotic cells from both C2 and 10TMyo cell lines were found to be mononucleated (49). These observations indicate that the apoptosis process may be involved in cellular turnover during transition of myoblasts to myotubes in order to get rid of extra or non-differentiated myoblasts. To investigate the involvement of calpain in PARP degradation and apoptosis process in L8 cultured cells, we examined the role that calpains play in influencing PARP concentration under three conditions: 1) transition of myoblasts to myotubes without inducers; 2) with apoptosis inducer A23187; and 3) with apoptosis inducer etoposide.

Effects of over-expressing CID on PARP concentration in presence of HS

Post-confluent L8/PC and L8/CID myoblasts were cultured with 2 % HS for 12 h, 24 h, and 48 h, respectively, with or without 24-h IPTG (5 mM) preincubation. At the end of treatment, cell lysates were extracted as described in the Materials and Methods section. SDS-PAGE was performed and Western blots were followed with anti-PARP antibody as a probe. Equal amounts of protein samples (50 µg) were loaded. We observed that over-expression of CID (indicated by IPTG supplement) increased intact PARP concentration by 33 % (Fig.3.1: Panel A) and 16 % (Fig.3.1: Panel B) when post-confluent L8/CID myoblasts were cultured with 2% HS medium for 12 h and 24 h, respectively, with IPTG incubation for 36 h (Panel A) or 48 h (Panel B), respectively. In contrast, intact PARP concentration did not change under same treatment in L8/PC myoblasts (Fig.3.1 Panels A and B). The increase in intact PARP concentration was not observed after 48-h induction of differentiation (Fig.31: Panel C). These observations
Figure 3.1 (Panel A, B, and C): Effect of CID on intact PARP (116 kDa) concentration following 12-h to 48-h induction of differentiation. Post-confluent L8/PC and L8/CID myoblasts were cultured with 2% HS medium for 12 h (A), 24 h (B), or 48 h (C) with or without 24-h IPTG preincubation. Cell lysates were extracted as described in the text. SDS-PAGE was performed and a Western blot was followed with anti-PARP monoclonal antibody.
Figure 3.1 (E): Representing scanning densitometry of data shown in Panels A through C of L8/CID cell line. PARP was stabilized by 33% following 12-h induction of differentiation, 16% following 24-h induction of differentiation. There is no differentiation between treatment and control after 48-h induction of differentiation.
indicate that calpains play a role in regulation of intact PARP concentration during the onset of myoblast differentiation.

**Effects of CID on intact PARP concentration following addition of 1 µM A23187**

The observations that calpain is involved in the regulation of intact PARP concentration during the onset of differentiation caused us to question whether calpain plays a role in PARP degradation induced by apoptosis. Calpain inhibitors have been reported to inhibit apoptosis in some cell lines. Ca++ ionophore (A23187), a mediator of apoptosis and stimulator of calpain activity, was used in this study.

Post-confluent L8/CID myoblasts were cultured with DMEM medium (serum deprivation) with addition of A23187 (1 µM) for 0, 12, 24 h, respectively, with or without 24-h IPTG (5 mM) preincubation. At the end of the treatments, cell lysates were extracted. SDS-PAGE was performed and a Western blot was followed with anti-PARP monoclonal antibody as a probe. Equal amounts of protein samples (50 µg) were loaded. We observed that intact (116 kDa) PARP concentration was reduced as a consequence of incubation with 1 µM A23187 for 12 h and 24 h, respectively (i.e. compare lanes 2 and 4 with lane 1, Fig. 3.2 (A)). However, over-expression of CID (inhibition of calpain) antagonized the effects of A23187 (see Figure 3.2 (A) lane 3 vs lane 2 and lane 5 vs lane 4). Scanning densitometry (Figure 3.2 (B) results show that inhibition of calpain reduced 10 % or 40 % of PARP degradation following 12- or 24-h incubation with A23187, respectively. These observations clearly indicate that inhibition of calpain prevents the destabilization of PARP caused by A23187 treatment.
Figure 3.2 (A): Effects of CID on intact PARP concentration following addition of 1μM A23187. Post-confluent L8/CID myoblasts were cultured with DMEM medium following addition of 1μM A23187 for 0, 12, or 24 h, respectively, in presence or absence of 24-h IPTG (5 mM) preincubation. Cell lysates were extracted (see text). SDS-PAGE was performed and a Western blot was followed with anti-PARP monoclonal antibody as a probe.
Figure 3.2 (B): Representing scanning densitometry of data shown in Panel A. PARP was degraded following 12-h or 24-h treatment with A23187, compared to control (0 hour). PARP was stabilized by 10% (12 hour) or 40% (24 hour) following over-expression of CID.
A

L8/CID

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Etoposide (300 nM)

IPTG (5 mM)

PARP (116 kDa)

Figure 3.3 (A): Effects of CID on intact (116 kDa) PARP concentration following 48 h incubation of etoposide. Post-confluent L8/PC and L8/CID myoblasts were cultured with DMEM medium containing 300 nM etoposide in presence or absence of 24-h IPTG (5 mM) preincubation. Cell lysates were extracted (see text). SDS-PAGE was performed and a Western blot was followed by using anti-PARP monoclonal antibody as a probe.
Figure 3.3 (B): Representing scanning densitometry of data shown in Panel A. PARP degradation occurs following 48-h treatment with etoposide, compared with 0-h treatment. Inhibition of calpain by CID was stabilized by 31%, compared with no inhibition of calpain.
Discussion

We have established a genetic approach to inhibit endogenous calpain activity by over-expressing calpastatin inhibitory domain (CID) in cultured cells. To determine the inhibition efficiency, we tested the over-expression of CID at the mRNA level by RT-PCR and examined effects of over-expressing CID on endogenous calpain activity by testing the prevention of fodrin breakdown products following A23187 treatment (see Chapter 1). We found that CID mRNA was over-expressed following addition of IPTG (5 mM) to L8/CID cells. Furthermore, over-expression of CID inhibited endogenous calpain activity as indicated by stabilization of fodrin, a typical calpain substrate (27). To determine whether calpain regulates PARP concentration in differentiating myoblasts, we tested (1) effects of over-expressing CID on intact PARP concentration on the onset of myoblast differentiation (post-confluent myoblasts cultured with 2% HS); (2) effects of over-expressing CID on intact PARP concentration following A23187 treatment; and (3) effects of over-expressing CID on intact PARP following etoposide treatment. Our data demonstrated that over-expressing CID increased intact PARP concentration in non-apoptotic cells and stabilized PARP concentration following A23187 and etoposide treatments (i.e., during apoptosis). These observations indicated that calpains are involved in regulation of PARP concentration in differentiating myoblasts.

Poly(ADP-ribose) synthesis, catalyzed by PARP, has been suggested to be involved in various physiological events, including DNA excision repair, DNA recombination, DNA replication, cell differentiation, cell growth and transformation (1), and apoptosis (10,11). Cleavage of PARP is a hallmark of apoptosis in many mammalian cells (10,11). Degradation of PARP in vitro by calpain has been observed and reported (19). To our knowledge, this is the first report that calpains are involved in regulation of PARP protein in living cells, which indicates that calpain may be involved in the control of PARP concentration. This opens up a new area for study of calpain’s physiological function.
Serum deprivation-induced apoptosis has been demonstrated (61,62). A23187- and etoposide-mediated apoptosis have also been reported in many cell lines (57-59, 63-65). Degradation of PARP has been observed in many mammalian cells (10-11). In this study, we observed that PARP protein level was reduced following serum deprivation plus A23187 or etoposide in differentiating myoblasts, indicating that apoptosis may occur upon degradation of PARP. Involvement of calpain in dexamethasone-induced apoptosis in thymocytes (33), TGF-β-induced apoptosis in hepatocytes (34), T cell receptor-associated apoptosis in lymphocytes (35), and activation-induced apoptosis in HIV-infected T lymphocytes (36) has been demonstrated and reported. Furthermore, involvement of calpain in serum deprivation-induced apoptosis in C3H/10T1/2 muscle satellite cells (66) and autopsy autolysis-associated apoptosis in cardiac myocytes (67) have been observed. Consequently, the observations that calpains are involved in steady-state poly(ADP-ribose) polymerase concentration in serum deprivation plus etoposide- or A23187-associated differentiating myoblasts leads us to believe that calpain may be involved in differentiation-associated apoptosis in L8 myoblasts.

Myogenesis-associated apoptosis has been demonstrated in differentiated myocardial muscle (46), neonatal skeletal muscle (43), skeletal myoblasts in response to injury (47), and wound-healing muscle (48). In many degradative and metabolic muscle diseases cells die without a marked inflammatory response (68). A potential mechanism for this is apoptotic cell death. It has been reported that myonuclear apoptosis is an early event in the pathology of dystrophin-deficient muscular dystrophy in the mdx mouse (69-71). However, the mechanism of apoptosis in myogenesis and muscle disease is barely known and key factors which are involved in the pathway have not been determined yet. It has been demonstrated that merosin, the predominant laminin variant in skeletal muscle basement membranes, appears to promote myotube stability by preventing apoptosis prior to myoblast fusion (49). Insulin-like growth factor-II has been reported to prevent apoptosis in differentiating myoblasts (72). However, roles of the ICE- or caspase-families, proven to play more important roles in mediating the process of apoptosis in general, have not been reported in skeletal muscle apoptosis, although it is known that
skeletal muscle undergoes apoptosis (i.e., mdx mouse muscle shows evidence of apoptosis (69-71)). Proteases responsible are not known with certainty. The observation that calpains participate in PARP degradation indicate a relationship (albeit a very indistinct relationship, based upon the very preliminary data presented here) to cell death. Roles of the individual calpains (i.e, µ-, m-, µ-/m- and p94) in control of PARP need now to be examined.
References


Chapter IV

Overall Summary

Muscle protein degradation is a significant determinant of muscle growth and muscle size. The first goal of this study was to investigate the role of calpains in skeletal muscle protein degradation in cultured cells. We have developed a genetic approach to inhibit endogenous calpain activity through over-expressing dominant negative m-calpain (DN), antisense m-calpain (AS) and calpastatin inhibitory domain (CID). We observed that, under conditions of accelerated degradation (serum withdrawal), inhibition of m-calpain through DN-m-calpain over-expression caused a 30% inhibition of total myotubular protein degradation whereas CID over-expression reduced degradation by 63%. These constructs did not significantly affect degradation in the presence of serum. These data indicate that calpains participate in the accelerated degradation associated with serum withdrawal. Inhibition of calpain also stabilized nebulin, a major structural protein of the sarcomere. These observations indicate that calpains play significant roles in muscle protein turnover. Finally, over-expression of antisense m-calpain caused a transient reduction in m-calpain concentration after which normal m-calpain concentration was quickly re-established. These observations indicate that m-calpain is a short half-life protein in muscle cells.

The second goal of this study is to investigate the role of calpain, a Ca+-dependent cysteine protease, in the mediation of PARP protein level in differentiating myoblasts. Poly(ADP-ribosyl)ation, catalyzed by PARP, is involved in various physiological events, such as DNA excision repair, DNA recombination, DNA replication, cell differentiation, cell growth and transformation (5), and apoptosis (86-90). A protease participating in PARP turnover could be a significant regulator to the events which PARP is involved. Further, a relationship between apoptosis and myofibrillar protein degradation via a common protease might suggest the basis for muscle wasting and atrophy which
characterize in many muscle diseases. We established a genetic approach to inhibit endogenous calpain activity through over-expressing calpastatin inhibitory domain (CID). We observed that (1) inhibition of calpain activity increased PARP concentration when post-confluent myoblasts were cultured with 2 % HS medium, an inducer of differentiation and (2) inhibition of calpain activity prevented PARP degradation induced by A23187 and etoposide in differentiating myoblasts. These data demonstrate that calpain is involved in regulation of PARP in cultured cells.
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Appendix

Identification of Discrete Steps in the Process of Muscle Cell Differentiation in Cell Culture

Jing Huang and Neil Forsberg

To submit to Differentiation
Abstract

The goal of this work was to understand more fully the control of the process of differentiation in cultured muscle cells. The rationale for this work was that we were interested in identifying the factors which lead to the gradual loss of differentiation ability of individual cell lines. L8 myoblasts were cultured at various densities on tissue culture dishes and interactions between individual clones were assessed. Of the clones which were studied, many lost their ability to differentiate and we focused our attentions on these. Non-differentiating myoblast colonies, when cultured adjacent to differentiation-competent colonies exhibited a variety of responses. First, some myoblasts associated with the periphery of differentiation-incompetent cells differentiated (i.e., myoblasts aligned then fused to form myotubes) when cultured adjacent to differentiated cells. These data indicated that the differentiated colony secreted factor(s) which induced the adjacent colony to differentiate. Second, in other colonies some differentiation-incompetent myoblasts aligned in preparation for fusion but never actually fused. These data indicate that alignment and fusion, while being integral to the process of differentiation, are independently regulated. While the myoblast-derived factor responsible for alignment and fusion was not identified in this study, we propose that the use of these cell lines could provide a useful strategy for its identification and for further examination of the control of muscle cell differentiation.

Key Words: myogenesis, L8 muscle cell, differentiation, myoblast, alignment, fusion


Introduction

Cultured muscle cells have been used extensively to study the process of differentiation. Differentiation in muscle cell lines is accompanied by remarkable phenotypic changes (formation of multi-nucleated myoblasts) and biochemical changes (expression of muscle-specific genes). In our laboratory, we have used L8 myotubes to study the process of myofibrillar protein degradation [1-3]. However, more-recently, we have also used this line to examine the roles of individual proteases in the process of remodeling during muscle cell differentiation [4]. When L8 myoblasts are cultured in mitogen-rich media (such as Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS)), they proliferate only. However, when they reach confluence and serum is switched to 2% horse serum (HS), they begin to differentiate [4]. Differentiation begins with myoblast elongation and alignment, fusion, formation of small myotubes and, finally, formation of large multi-nucleated myotubes in which several hundred myonuclei may cluster in well-defined regions of the cell. This process requires 4-5 days in culture and is thought to be stimulated by the removal of fibroblast growth factor (FGF) from culture media [5,6,7,8]. FGF has been postulated to maintain myogenic family proteins (e.g., myogenin) in a phosphorylated state that makes them unable to complex with E2 proteins and thereby initiate muscle-specific gene expression. Often, the ability of individual cell lines to differentiate declines following multiple passages of myotubes. Hence, it is necessary to reclone cell lines in order to maintain lines which maintain high differentiation rates. This loss of the ability to differentiate has never been fully understood. But, clearly, biochemical or genetic changes must occur in senescing cell lines which prevent differentiation from occurring. It is during the recloning process that we have made several observations which illuminate the mechanisms which coordinate cell-to-cell contact, alignment and fusion; events which precede the physical aspects of myoblast differentiation and which may be lost during passage and thereby prevent differentiation.
The goal of this study was to understand more thoroughly the interactions of L8 myoblast clones in culture. Specifically, we studied the interactions of cells within clones and how these interactions facilitated the differentiation process. We also examined the interactions between adjacent clones to determine whether factors from individual colonies were produced which might influence differentiation of adjacent clones. From our analysis, we have determined that a differentiation signal originates from the center of each clones of myoblasts. This signal is diffusible and transmutable. The adjacent non-differentiating colony could be induced to differentiate. We also identified the discrete steps in the process of muscle cell differentiation in cell culture.
Methods

Rat L8 myoblasts (American Type Tissue Collection, Manassas, VA) were cultured in DMEM (Gibco, Gaithersberg, MD) with addition of 1000mg/l D-glucose, 584 mg/L L-glutamine, 110 mg/l sodium pyruvate, 4 mg/l pyridoxine hydrochloride, 3.7 g/l sodium bicarbonate, 100 U/ml penicillin-streptomycin (Gibco) and 10% (v/v) fetal bovine serum (characterized FBS, Hyclone, Logan UT). Cells were trypsinized when they reached 70% confluence and replated to a density as low as 20-30 cells per plate. Ten plates were prepared for each density. The medium was changed every other day for myoblasts. The photographs were taken using a Nikon (Model N6000) inverted stage microscope using 40X magnification.

For the purpose of recloning cell lines, when individual clones were large enough to be visualized without a microscope, they were transferred and cultured in 24-well, 12-well, 6-well, 50 mm, and, finally, 100 mm plates, sequentially. For each clone, half were stored in a -80 °C freezer and another half continued in culture to assess differentiation. Individual clones were monitored closely following plating. For the purpose of investigating the interaction between clones, when individual clones were large enough to be visualized without a microscope, they were cultured with 2 % HS and interactions between cells within a single clones and between adjacent clones were closely monitored. Only interactions which were observed repeatedly are presented in this manuscript.
Results and Discussion

During the process of recloning, we found that some of the new colonies of cells differentiated readily (e.g. Figure 1) while other colonies had completely lost the ability to differentiate and, instead, when challenged by mitogen-poor medium, simply maintained a confluent population of myoblasts (e.g., Figure 2). Clearly, some factor(s) had been lost from non-differentiating colonies which allowed the normal process of differentiation to proceed. We reasoned that further study of this phenomenon might prove useful in identifying the factor(s) which coordinated differentiation or a factor(s) which was lost in certain cell lines which was also important to the process of differentiation.

We studied the process of differentiation of individual colonies and first noted that, among colonies which retained ability to differentiate, differentiation always began in the center of the colony. The process then radiated from the center (e.g., Figure 3) of the colony to the periphery (Figure 4). These observations indicate one of two possibilities: 1) there is a myoblast-derived differentiation signal which exists at highest concentration at the center of the colony or 2) cells at the center of the colony are "older" than the nascent cells of the periphery and, therefore, are chronologically programmed to initiate the process of differentiation. By mass action, the first possibility would be predicted to occur at the center of a population of myoblasts in which each cells produced the factor in similar amounts.

To distinguish between these two possibilities, we reasoned that, if a paracrine-acting differentiation signal was produced by a differentiation-competent colony, it should stimulate differentiation of myoblasts on the periphery of nearby colonies. To assess this, we cultured myoblasts at higher density (100 cells per plate) to enable some colonies to grow in closer proximity to one another. We found that some fully-differentiated clones were able to induce changes in adjacent colonies which were quite remarkable and which provide evidence for the independent regulation of two critical events which precede differentiation. First, in some cases, a differentiation-competent colony (A) was able to induce differentiation (alignment and fusion) of adjacent differentiation-incompetent
colonies (B) (e.g., Figure 5). In this case, differentiation was induced within the peripheral layer of myoblasts instead of the center of the clone. Our interpretation of this observation was that such clones lacked an auto-generated differentiation signal and that this signal was provided by the adjacent differentiation-competent colony. This provides clear evidence of paracrine control of two processes involved in differentiation of muscle cells: alignment and fusion. Others have shown that insulin, IGF-I and -II and N-, M-cadherin are produced by cultured myoblasts and stimulate differentiation [9-15]. The myoblast-derived factor(s) inferred by our observations could be IGFs, N-, M-cadherin or other uncharacterized factors.

Alignment and fusion may be viewed as integral events leading to differentiation; however, additional observations indicated that differentiation-incompetent myoblasts could become defective in one or both of these processes. For example, culture of a differentiated colony (A) adjacent to some differentiation-incompetent colonies caused the myoblasts of the adjacent colony (B) to align but not fuse (e.g., Figure 6; see arrow). This suggests that ability to align, while a requisite for fusion, does not automatically lead to fusion and that differentiation-competent cells produce an alignment signal which initiates a multi-step process leading to complete differentiation. Additional events must be regulated following alignment to allow fusion to occur. Some of our clones clearly had lost this regulatory feature. To support to our observation, It has been reported that M-cadherin (14,15) and merosin (19) are involved in myoblast fusion of skeletal muscle.

Finally, we also found differentiation-incompetent clones which, despite having proximity to fully-differentiated colonies, were unable to align or to fuse (Figure 7) These differentiation-incompetent colonies appear to have lost their ability to recognize the alignment signal produced by the adjacent differentiation-competent colony. This alignment signal, which may be one of the well-characterized regulators of muscle cell differentiation, may also be the signal which induces fusion and subsequent differentiation. The conclusions of this work are that several steps are involved in the coordination of myoblast differentiation. First, differentiation-competent myotubes secrete an alignment factor(s) which induces adjacent myoblasts to align and to fuse. Some cell lines lose their
ability to differentiate by losing ability to respond to this factor. Several endocrine factors, such as IGFs [16-18], have been implicated in this control and could serve as the basis for our observations. Second, fusion does not automatically follow alignment. While a single factor may regulate both alignment and fusion, we recovered clones capable of aligning but not fusing. Clearly, some of our cell lines lost their ability to fuse but retained their ability to align. These data indicate that alignment and fusion are related but independently-regulated processes. Loss of control at any of these steps could serve as the basis for the loss of ability of a single clone of muscle cells to differentiate. We propose the use of these clones could help to unravel the intricate processes intrinsic to muscle cells which lead to a differentiated phenotype.
A.1: A clone with fully-differentiated myotubes.
Figure A.2: A clone with undifferentiable myoblasts.
Figure A.3: A clone that start to differentiate from its center.
Figure A.4: A differentiating clone that radiated from the center of the colony to the periphery
Figure A.5: One fully-differentiated clone (A) initiates adjacent non-competent clone (B) to differentiate.
Figure A.6: A differentiating myoblast clone (A) initiates adjacent non-competent myoblast clone (B) to elongate.
Figure A.7: A non-competent myoblast clone could not be effected to differentiate by an adjacent fully-differentiated myotube clone although they are close to each other.
References


