AN ABSTRACT OF DISSERTATION OF

Juntipa Purintrapiban for the degree of Doctor of Philosophy in Animal Science presented on July 26, 1999.

Title: Coordination of Protease Systems on Muscle Protein Degradation and Identification of Calpain Substrates Using the Yeast Two-Hybrid System.

Abstract approved: _____

Forsberg, Neil E.

The first goal of this study was to evaluate the roles of different intracellular proteolytic systems in accelerated skeletal muscle protein degradation. Total, myofibrillar and non-myofibrillar proteolysis were determined in cultured L8/CID muscle cells. Calpain proteolysis was assessed by over-expressed calpastatin inhibitory domain (CID). Lysosomal proteolysis was determined in the presence of methylamine. Ub-proteasome proteolysis was measured by inhibiting proteasome activity with Z-Leu-Leu-Leu-H (aldehyde) (ZLLL). Accelerated protein degradation, induced by serum withdrawal, increased myofibrillar and non-myofibrillar protein degradation by 31 and 38%, respectively. Inhibition of all protease systems reduced total protein degradation by approximately 67%. Ub-proteasome and lysosomal pathways accounted for most protein degradation (62% and 40%, respectively). The lysosome contributed non-selectively to the myofibrillar protein (27%) and non-myofibrillar protein (27%) turnover. The Ub-proteasome system appears mainly responsible for the myofibrillar

pool degradation (10%) and has a slight effect in non-myofibrillar protein degradation (5%). The calpain system has a slight effect on overall proteolysis (20%). However, the calpain plays an important role in initiating muscle protein degradation.

Inhibition of the lysosomal and proteasome pathways stabilized tropomyosin, desmin, filamin, α-actinin, and dystrophin. The accumulated levels of desmin and dystrophin increased by additional CID overexpression, indicate the coordination of the protease systems in degradation of individual proteins as well as the role of calpain in initiating protein degradation.

Numerous studies have shown that calpain cleaved a variety of substrates *in vitro*. The second goal of this study was to identify the actual substrates of m-calpain in skeletal muscle. A dominant negative m-calpain was used as a bait for screening human skeletal muscle cDNA libraries in the yeast two-hybrid system. Four partial cDNAs encoding creatine kinase, glycogen phosphorylase, Ca^{2+}-ATPase and nebulin-related protein were identified as m-calpain binding proteins. Purified creatine kinase and glycogen phosphorylase were cleaved by m-calpain in an *in vitro* assay. These results suggest that creatine kinase and glycogen phosphorylase interact with m-calpain and m-calpain may regulate the degradation of creatine kinase and glycogen phosphorylase and possibly regulate their activities. To date, we have not yet evaluated whether Ca^{2+}-ATPase and nebulin-related protein also serve as substrates for calpain.

Coordination of Protease Systems on Muscle Protein Degradation and Identification of Calpain Substrates Using the Yeast Two-Hybrid System

by

Juntipa Purintrapiban

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Doctor of Philosophy

Presented July 26, 1999

Commencement June 2000

Doctor of Philosophy dissertation of Juntipa Purintrapiban presented on July 26, 1999

APPROVED:

Redacted for Privacy

Major Professor, representing Animal Science

Redacted for Privacy

Head or Chair of Department of Animal Sciences

Redacted for Privacy

Dean of Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Redacted for Privacy

Juntipa Purintrapiban, Author

ACKNOWLEDGMENT

I sincerely thank my major professor, Dr. Forsberg for his trust, encouragement, support and friendship throughout my program. I would like to think Dr. Froman and Dr. Davis for their support in many ways to help me accomplish my studies. I would also like to thank Dr. Whanger, Dr. Sharrow, Dr. Gamble and Dr. Torres for serving on my program committee.

I greatly appreciate my colleagues, Mike Beilstein, Jing Huang, Yuhg-Hae Kim, Ying-Yi Xiao, Yoji Ueda, Pai-Yen Wu and Mei-Chuan Wang, for their friendship, encouragement and scientific discussions. I give special thanks to Mei for her assistance in many ways. I could not have accomplished so much without support from each of them.

Finally, I give thanks to all the staff in the Department of Animal Sciences and Oregon State University for providing an excellent learning and working environment during my studies.

TABLE OF CONTENTS

Page

INTRODUCTION.....1

LITERATURE REVIEW.....4

MATERIALS AND METHODS.....21

RESULTS.....38

DISCUSSION.....69

CONCLUSION.....79

BIBLIOGRAPHY.....80

LIST OF FIGURES

Figure		Page
1.	Autolytic activation of calpain	7
2.	Schematic diagram showing the domain structure of bovine cardiac calpastatin.	17
3.	The two-hybrid system	20
4.	Expression vectors in the yeast two-hybrid system	27
5.	Schematic diagram of using the yeast two-hybrid system to screen cDNA library for an interaction between two proteins	32
6.	Time-dependent response of total protein degradation following serum withdrawal in L8/CID myotubes	40
7.	Effect of IPTG on total protein degradation at 6 hours following serum deprivation in L8/CID myotubes	41
8.	Effect of IPTG on total protein degradation at 12 hours following serum deprivation in L8/CID myotubes	42
9.	Effect of IPTG on total protein degradation at 24 hours following serum deprivation in L8/CID myotubes	43
10.	Effect of over-expressed CID, proteasome and lysosomal inhibitors on m-calpain concentration in L8/CID myotubes	44
11.	Effect of methylamine on total protein degradation at 12 hours following serum deprivation in L8/CID myotubes	46
12.	Effect of methylamine on total protein degradation at 24 hours following serum deprivation in L8/CID myotubes	47
13.	Effect of ZLLL on total protein degradation at 12 hours following serum deprivation in L8/CID myotubes	48
14.	Effect of ZLLL on total protein degradation at 24 hours following serum deprivation in L8/CID myotubes	49

LIST OF FIGURES (CONTINUED)

Figure — Page

15. Effects of 24 hr-treatment of various protease inhibitors on total protein degradation following serum withdrawal in L8/CID cell lines..... 51

16. Effects of 24 hr-treatment of various protease inhibitors on stability of non-myofibrillar proteins in L8/CID cell lines..... 53

17. Effects of 24 hr-treatment of various protease inhibitors on stability of myofibrillar proteins in L8/CID cell lines..... 54

18. Effect of over-expressed CID, proteasome and lysosomal inhibitors on LacI concentration in L8/CID myotubes..... 56

19. Effect of over-expressed CID, proteasome and lysosomal inhibitors on desmin (panel A) and dystrophin (panel B) concentration in L8/CID myotubes..... 57

20. Effect of over-expressed CID, proteasome and lysosomal inhibitors on α-actinin (panel A) and filamin (panel B) concentration in L8/CID myotubes.. 58

21. Effect of over-expressed CID, proteasome and lysosomal inhibitors on tropomyosin concentration in L8/CID myotubes..... 59

22. Expression of m-calpain/GAL4-BD hybrid proteins in the yeast strain Y 190.. 61

23. PCR-amplified products of the human skeletal muscle cDNA library plasmids derived from two-hybrid screening with m-calpain..... 64

24. Expression of library/GAL4-AD hybrid proteins in the yeast strain Y 190 ... 65

25. SDS-PAGE (10%) showing the effects of digestion by m-calpain on creatine kinase..... 67

26. SDS-PAGE (7.5%) showing the effects of digestion by m-calpain on glycogen phosphorylase..... 68

27. Schematic diagram showing the structure and protein composition of costameres in striated muscle relative to Z-disks and the myofibrillar lattice... 73

28. Possible schematic diagram for linking the terminal actin filaments of myofibrils to the cell membrane..... 76

LIST OF TABLES

Table Page

1. Yeast mating partners to eliminate false positives 33

2. Interaction between calpain and proteins in skeletal muscle cDNA library 62

DEDICATION

This Doctoral Dissertation is dedicated to:

My family: my mother, Srinuen; my sisters, Vimonrat and Sunaiyana; my brother, Trirong, for their love, encouragement and support.

The Royal Thai Government and Prince of Songkla University for providing a scholarship.

COORDINATION OF PROTEASE SYSTEMS ON MUSCLE PROTEIN DEGRADATION AND IDENTIFICATION OF CALPAIN SUBSTRATES USING THE YEAST TWO-HYBRID SYSTEM

INTRODUCTION

The rate of muscle protein turnover has significant effects on both the rate and efficiency of skeletal muscle growth. Modulating protease activity in muscle could have major effects on the rate of muscle protein growth. It has been estimated that 15 to 25% of food ingested by domestic animals is used to replace muscle protein that is degraded during metabolic turnover (Young et al., 1975). Therefore, decreasing the rate of protein degradation would result directly in increased efficiency by which animals convert nutrients into muscle. In addition, several studies have demonstrated that growth promotants in livestock stimulate muscle growth through an inhibition of muscle protein degradation (Yang and McElligott, 1989; Forsberg et al., 1989; Parr et al., 1992; Pringle et al., 1993).

Protein degradation in a tissue is mediated by a mixture of proteolytic systems which, presumably, act in concert to mediate the complete disassembly and digestion of proteins to amino acids. The three major proteolytic systems are the lysosomal, Ca^{2+}-dependent and ubiquitin (Ub)-proteasome pathways. Atrophying muscle is the best sample of the dramatic changes that can occur in muscle protein mass. The most common causes of skeletal muscle atrophy are disuse and denervation, systemic acidosis, systemic metabolic disturbances, glucocorticoid excess and starvation. Exercise-induced damage, systemic sepsis, and burn injury are also able to stimulate multiple proteolytic pathways in skeletal muscle (Belcastro et al., 1998; Tiao et al.,

1994; Hummel et al., 1998; Fang et al., 1995; Voisin et al., 1996; Mansoor et al., 1996). In such pathological situations, rapid atrophy of skeletal muscle is largely the result of an increased rate of protein degradation that may be mediated by the Ub-proteasome system. However, increased activities of calpain and lysosomal proteases are also observed in these situations (Wing and Goldberg, 1993; Tischler et al., 1990; Baracos et al., 1995; Weinstein et al., 1997). Hence, there is uncertainty regarding which of the proteolytic systems plays the key rate-limiting event.

Although there is abundant evidence that each protease system plays a role in a large number of physiological processes, the specific roles of each protease in muscle protein degradation have not been established. It is important to learn what enzymes are involved in degrading particular pools of proteins and individual protein to be able to understand their regulation.

Calpain is a multifunctional protease discovered in 1964 (Guroff and Guroff, 1964; Meyer et al., 1964). The structural and enzymatic properties of the two most common isoforms (μ- and m-calpain) are well characterized. Nevertheless, the physiological function of calpain is not yet well understood. Several lines of evidence indicate that the calpain system plays an important role in prenatal myoblast fusion and proliferation of myogenic cells in developing muscle tissue and in initiation of myofibrillar protein turnover in mature muscle cells (for reviews see Wang and Yuen, 1999). Calpain's ability to process proteins (instead of completely digesting them) indicates its significant role in a variety of physiological events. The putative role of calpain is in remodeling/disassembly of cytoskeletal and plasma membrane

interactions (Goll et al., 1992a). We know many cytosolic and myofibrillar proteins are substrates of calpain. However, there is limited information on the regulation of the turnover of these substrates *in vivo*. Based on this, it is of very much interest to assess the specific roles of calpain in the turnover of individual protein in living cells.

The objective of our research was to understand the functions of the proteases (mainly calpain) in skeletal muscle. The first of two specific aims was to learn how the muscle protein degradation is regulated in skeletal muscle. We were specifically interested in learning the role of each protease in degrading particular pools of protein and specific proteins. In this study, we used cultured rat skeletal muscle cells (L8 cell line) as a model and assessed the effects of protease inhibitors on muscle protein degradation. The second aim was to determine the actual substrates of m-calpain in skeletal muscle and characterize the role of m-calpain in regulating those proteins. We used the yeast two-hybrid strategy to identify muscle m-calpain binding proteins from a skeletal muscle cDNA library.

Results from this study may be used to:

1. Establish the role of the protease system responsible for muscle protein degradation.
2. Regulate the protease system to increase muscle mass or to enhance the efficiency of muscle growth.
3. Define substrates of calpain in muscle cells.
4. Determine the potential roles of the calpain system in muscle cells and muscle pathologies.

LITERATURE REVIEW

Skeletal Muscle and Muscle Proteins

Skeletal muscle is the best known and understood muscle of the four types of muscle tissue. In addition to its role in movement, it is also an important source of nutrition during protein and energy deprivation (Scornik et al., 1997). Sarcomeres, the unit of muscle physiological function, comprise the major mass in muscle and permit contraction for force generation and transmission (Bates and Millward, 1983). The individual proteins of the myofibrils are continuously undergoing degradation and synthesis. Both of these processes are dependent on the age of animal, the subtype of the muscle, activity, health and endocrine states (Bates and Millward, 1983; Allen 1988; Millward 1980).

Muscle fibers (skeletal muscle cells) originate from the embryonic mesoderm. The precursors of muscle cells, myoblasts, fuse with one another to form multinucleated skeletal muscle cells. Once formed, a skeletal muscle cell is generally retained for the entire lifetime of the animal. Myofibrillar proteins organized as contractile elements (myofibrils) of the muscle cell and represent up to 80% of total muscle proteins (Goll et al., 1991). The remaining proteins are classified as non-myofibrillar proteins which include other sarcoplasmic proteins, nuclear and membrane proteins. The non-myofibrillar proteins are easily extracted from muscle with low salt buffer whereas the myofibrillar proteins are soluble in high salt buffer.

The arrangement of thick and thin filament gives skeletal muscles a striated appearance (Fleischer and Inui, 1989). The dark or A band of sarcomere contains a set of parallel thick filaments, which are composed almost entirely of myosin. The light or I band contains a set of the thin filaments that are anchored to the Z disk. The center of the sarcomere where actin and myosin do not overlap is defined as the H band. The thin filaments consist mainly of actin and two other major proteins, tropomyosin and troponin. The troponin-tropomyosin complex regulates muscle contraction by varying the access of myosin heads to their actin binding sites in response to the concentration of Ca^{2+} (Farah and Reinach, 1995). Troponin consists of three subunits: TnC, a Ca^{2+}-binding calmodulin homolog; TnI, which binds actin; and TnT, which binds tropomyosin. During muscle contraction, the think and thin filaments maintain constant lengths whereas Z lines move closer together. Sliding of the thin filaments over the thick filaments during muscle contraction is produced by breaking and re-forming of the cross-bridge between actin and myosin. This cyclic process is driven by ATP hydrolysis. Each single cycle shortens muscle by about 1%.

Many proteins, which are less abundant but perform very important role in muscle cell, are in the non-myofibrillar fraction. This fraction is rich with glycolytic enzymes, proteins involved in signal transduction and differentiation control. The Z-disk contains several fibrous proteins which are required for myofibril assembly and stabilization (Voet and Voet, 1995). For instance, α-actinin attaches thin filaments to the Z disk. Desmin and vimentin localize at the Z disk periphery. Titin links thick filaments to Z disk and confers passive elasticity. C-protein and M-protein associate

with the M disk, which arises from the local enlargement at the center of the thick filaments. Nebulin winds along the length of a thin filament, thereby controlling this length. Dystrophin is associated with muscle plasma membrane.

Calpain Structure and Properties

Calpain is a calcium-activated cysteine protease that was originally identified in rat brain (Guroff and Guroff, 1964). Two isoforms (μ- and m-calpain) are well characterized and several tissue-specific isoforms have been reported (Sorimachi et al., 1997). μ- and m-calpain, which require a micromolar and a millimolar concentration of Ca^{2+} for activation, respectively, exist ubiquitously in mammalian tissues. Both isoforms are heterodimers and consist of a unique catalytic (80-kDa) subunit and an identical regulatory (30-kDa) subunit. The large (80-kDa) subunit can be divided into four domains (I-IV, Figure 1). Domain II is the cysteine protease domain and its active site residues are similar to other cysteine proteases such as papain or cathepsins B, L, or S. Domain IV is a calmodulin-like domain containing five sets of E-F-hand sequences that are potential Ca^{2+}-binding sites. The functions of Domains I and III are less clear. However, the N-terminal region of Domain I is removed during autocatalytic activation and could play a role in inhibiting the enzyme or preventing access of substrate to the active site (Saido et al., 1993). Domain III contains two sets of E-F-hand motifs. The small (30-kDa) subunit comprises two domains (V and IV'). The N-terminal region, Domain V, is glycine-rich and possibly interacts with membrane phospholipids to localize or stabilize the enzyme complex

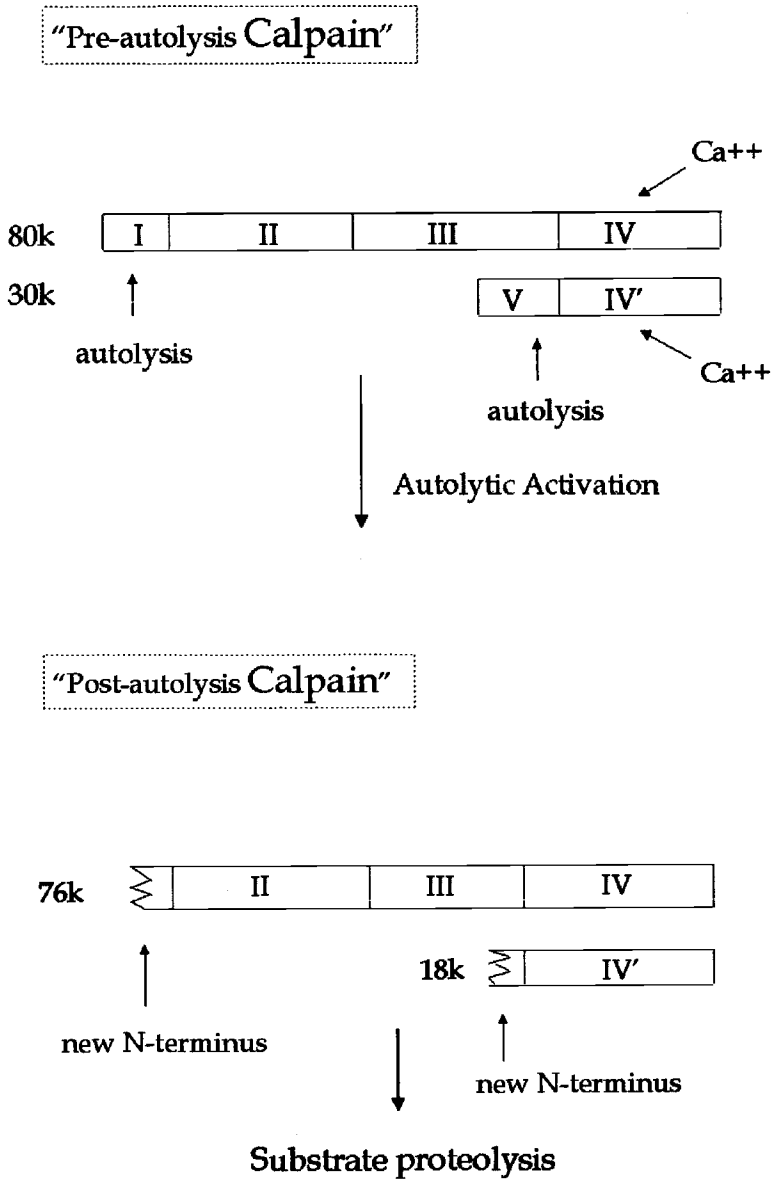


Figure 1. Autolytic activation of calpain. Both large and small subunits undergo autolysis upon activation, generating new N-termini. Substrate proteolysis follows autolysis.

(Sorimachi et al., 1990). The C-terminal portion, Domain IV', also contains five potential Ca^{2+}-binding sites.

Several tissue-specific calpains have been reported. The first discovered tissue-specific calpain is p94. This is expressed exclusively in skeletal muscle. It possesses three unique additional segments not found in other calpain species (NS, IS-1, IS-2) and does not associate with the conventional calpain small subunit (Kinbara et al., 1998). Muscle-specific p94 is activated and expresses its proteolytic activity at physiological Ca^{2+}concentration. More recently, an alternative spliced variant of p94, Lp82, was discovered in lens (Ma et al., 1999). nCL-2 is another calpain homologue which co-exists predominantly in the stomach with nCL-2', its alternatively spliced molecule. This spicing variant lacks the Ca^{2+}-binding domain and most of Domain III (Sorimachi et al., 1993), suggesting that the function of nCL-2' is independent of calcium. Various atypical calpain homologues also exist in lower organisms such as insects, nematodes, fungi and yeast (Ono et al., 1998; Sorimachi et al., 1997). Those proteins share conserved structures similar to the conventional calpain large subunit (Domains I and II). Interestingly, most of these homologues contain other unique domains which are likely responsible for the many unique functions they possess.

Regulation of Calpain Activity and Autolysis

Calpain autolysis is necessary for expression of its catalytic activity. Autolysis of calpain is initiated upon the increase of intracellular Ca^{2+} concentration. Autolysis removes the N-terminal 19 or 27 (m- or μ-calpain) amino acids from the 80-kDa

polypeptide and the N-terminal 91 amino acids from the small polypeptide (Suzuki 1990). The Ca^{2+} concentration required for half-maximal activity of calpain measured *in vitro* ranges from 3-50 μM for μ-calpain and 400-800 μM for m-calpain (Cong et al., 1989; Kapprell and Goll, 1989). The Ca^{2+} concentration required for various properties of calpain is significantly higher than the physiological Ca^{2+} concentration in the cells (Harkins et al., 1993). Increases in intracellular free [Ca^{2+}] up to 1-2 μM result in increased calpain activity (Hong et al., 1994; Turner et al., 1991) indicating that calpain responds to the fluctuation of free Ca^{2+} and does not require the high Ca^{2+} concentration indicated in *in vitro* assays. When Ca^{2+} concentration increases, translocation of calpain to the membrane was observed following by calpain activation (Saido et al., 1993). The autolyzed form is then released from the membrane (Pontremoli et al., 1985). N-terminal modification enhances Ca^{2+} sensitivity of calpain since the autolyzed forms apparently require less Ca^{2+} concentration for their activity. Binding to phospholipids in the cell membrane lowers the Ca^{2+} concentration required for calpain autolysis (Goll et al., 1992b). And, it was shown that calpain also binds to proteins in erythrocyte membranes (Kuboki et al., 1990) and in membrane vesicles (Inomata et al., 1989). Thus, membrane proteins, as well as phospholipids, are thought to provide important interaction sites. Recently, the membrane-associated activator specific for μ-calpain and m-calpain was detected in rat brain and skeletal muscle, respectively (Melloni et al., 1998; Michetti et al., 1991). The activator competes with calpastatin for the binding of the catalytic 80-kDa subunit, increasing its Ca^{2+} sensitivity and promoting the dissociation of calpain's heterodimeric structure.

It has been postulated that 30-kDa subunit acts as an inhibitor or pseudo-substrate. The dissociation of calpain into subunits happens as a consequence of autolysis and in the presence of Ca^{2+}. The dissociated large subunit alone retains full catalytic activity (Yoshizawa et al., 1995). The large subunit expressed in the bacculovirus/Sf9 cell system is fully active without the co-expression of the small subunit (Vilei et al., 1997). Therefore, the small subunit is important but not necessary for calpain activation; it may contribute to the proper folding of the large subunit.

The activity of calpain is also regulated by its endogenous inhibitor, calpastatin. Calpastatin is expressed ubiquitously in mammalian cells. Two forms of calpastatin (muscle type and erythrocyte type) exist from a result of alternative splicing of a single calpastatin transcript (Geesink et al., 1998). Under physiological conditions in which Ca^{2+} concentration is lower than those required to initiate proteolytic activity, calpastatin binds to calpain and completely inhibits all isozymes of calpain in a substrate-competitive manner (Kawasaki and Kawashima, 1996). One molecule of calpastatin can interact with four calpain molecules. The interaction of calpain and calpastatin is calcium-dependent (Kapprell and Goll, 1989).

Role of Calpain in Muscle Growth and Muscle Pathologies

Calpain is a housekeeping enzyme that is expressed constitutively. Hence, it performs common functions in all cells. These functions include embryonic myoblast fusion and differentiation (Barnoy et al., 1998; Balcerzak et al., 1998), cytoskeletal protein degradation and remodelling, membrane protein degradation (Saido et al.,

1994), activation of protein kinase C isoforms and degradation of membrane-bound hormone receptors (Goll et al., 1989). On the other hand, calpain analogues with tissue-specific expression patterns indicate that they are pivotal to tissue development, maintenance, or function (Sorimachi et al., 1989). Calpain is also considered to be a regulator of calcium-dependent cell functions (Goll et al., 1992a; Suzuki et al., 1995). In skeletal muscle, calpain initiates degradation of the sarcomeric complex by degrading proteins such as titin, nebulin, desmin, C-protein, M-protein, filamin and certain Z-disk proteins. This releases thick and thin filaments from the surface of myofibrils for digestion by other proteases (Solomon and Goldberg, 1996). Proteasome, the major cytosolic proteolytic mechanism, has no effect on proteins in intact myofibrils and intact myofibrils have never been observed in lysosomal structures, even in rapidly atrophy muscle (Solomon and Goldberg, 1996; Koomaraie 1992). However, both protease systems rapidly degrade individual myofibrillar proteins. Indeed, immunocytochemistry has revealed that 5% of muscle calpain is associated with the Z-disk and it has been proposed that this fraction accounts for the normal turnover of myofibrillar proteins (Goll et al., 1992a; Ishiura et al., 1980).

Calpain is also involved in normal muscle skeletal growth. Administration of various β-adrenergic agonists to animals results in a 10-30% increase in rate of accumulation of muscle mass (Yang and McElligott, 1989). Muscle calpastatin activity is significantly increased upon exposure to beta-agonists (Forsberg et al., 1989; Parr et al., 1992; Pringle et al., 1993). Some studies have found that m-calpain activity increases whereas muscle μ-calpain is decreased or unchanged (Higgins et al.,

1988; Koomaraie and Shackelford, 1991; Wang and Beermann, 1988). These results suggest that β-adrenergic agonists increase rate of skeletal muscle growth by decreasing rate of protein degradation via a calpastatin-dependent event.

Calpain is activated when cells are injured and cytosolic Ca^{2+} rises. Therefore, calpain plays an important role in tissue injury, necrosis and autolysis (Goll et al., 1992a). Calpain is also involved in numerous pathological events such as stroke, brain trauma, Alzheimer's disease, cardiac ischaemia, muscle dystrophy, aging, sepsis, cataract, thrombolic platelet aggregation, arthritis and malaria (Saido et al., 1994; Wang and Yend, 1994; Wang and Yuen, 1999). Recent investigations of calpain activation response to muscle injury have provided additional evidence of calpains'involvement in membrane reorganization, myofibril dissolution and the regeneration of replacement fibers following muscle injury and inflammatory response (Belcastro et al., 1998). More recently results have indicated a key role for calpain in apoptosis (Villa et al., 1998).

Calpain Substrates

In vitro, calpain cleaves a variety of substrates. These include cytoskeletal proteins (spectrin, integrin, fibronectin, desmin, vimentin, cadherin, talin, ankyrin, fodrin, filamin); myofibrillar proteins (troponin, tropomyosin, myosin light chain kinase, nebulin, C-protein, M-protein); soluble and membrane-associated enzymes (protein kinase A, protein kinase C, phospholipase C); receptor proteins (EGF receptors, ryadodine receptor); cytokines (interleukin 1α); transcription factors (Fos, Jun) and lens proteins (crystallins) (Potter et al., 1998; Belcastro et al., 1998; Shoshan-Barmatz et al.,

1994; Kawasaki and Kawashima, 1996). Calpain does not degrade proteins to small peptides or amino acids. Rather, cleavage occurs at specific sites, leaving large polypeptide fragments with altered physiological properties.

Although numerous proteins are cleaved by calpain *in vitro*, there is limited information on calpain regulation of those substrates *in vivo*. Shuster and Herman (1995) demonstrated that ezrin, the ERM protein located at the actin-membrane interface, is an *in vivo* substrate of μ-calapin. Ezrin is markedly increased in NIH-3T3-derived cell lines that stably overexpressed calpastatin (Potter et al., 1998). Inhibition of calpain by overexpression of calpastatin inhibitory domain and dominant-negative m-calpain in L8 cell lines also stabilized nebulin and fodrin (Huang and Forsberg, 1998). Studies by Sorimachi and colleagues (Sorimachi et al., 1995; Kinbara et al., 1998), using yeast two-hybrid approach to demonstrate protein-protein interaction *in vivo*, identified titin/connectin as p94-binding proteins. In addition, Shinozaki et al. (1998) demonstrated that the presenilin 2 loop domain (PS 2, a gene responsible for the early-onset familial Alzheimer's disease) binds to μ-calpain and may, therefore, be a calpain substrate.

The Protease Systems and Cellular Protein Degradation

The rate of protein degradation in a tissue is dependent upon physiologicalstate and is mediated by a mixture of proteolytic systems which, presumably, act in concertto mediate the complete disassembly and digestion of proteins to amino acids. The turnover of skeletal muscle proteins, which are an important source of nutrition during

protein and energy deprivation (Scornik et al., 1997), is regulated by three major proteolytic systems: the lysosomal, Ca^{2+}-dependent and Ub-proteasome pathways. The Ub-proteasome pathway was first demonstrated in the late 1970s and shown to selectively catalyze breakdown of abnormal and short-lived proteins (Goldstein et al., 1975; Etlinger and Goldberg, 1977). However, this system is also responsible for the turnover of the long-lived proteins that comprise the vast majority of intracellular protein (Rock et al., 1994). In this pathway, proteins are targeted for degradation by covalent ligation to ubiquitin and degraded by the 26S proteasome complex which digests proteins to small peptides (Kisselev et al., 1998). In catabolic conditions such as uremia, activity of the Ub-proteasome pathway increases. This is accompanied with increased levels of ubiquitin mRNA and mRNAs encoding multiple subunits of the proteasome and results in degradation of muscle proteins (Ding et al., 1997). The Ub-proteasome pathway is also activated in response to starvation, sepsis, muscle denervation, cancer, thermal injury and acute diabetes (Temparis et al., 1994; Wing and Goldberg, 1993; Ding et al., 1997; Wing et al., 1995; Baracos et al., 1995; Tiao et al., 1994).

Lysosomal proteases (cathepsins) are known to degrade endocytosed proteins. Most of the lysosomal proteases are cysteine proteases with the exception of cathepsins G and cathepsins A (serine proteases) and cathepsins D and cathepsins E which are aspartate proteases (Otto and Schirameister, 1997). Exogenous and endogenous proteins, which are taken up by the cell via endocytosis or autophagocytosis, are transported in the form of endosomes or autophagosomes and finally directed to the

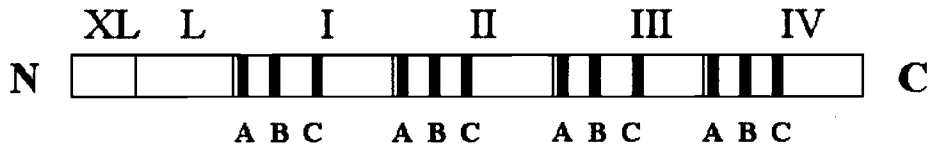
lysosomal pathway by fusion of these vesicles to lysosomes. Proteins can be degraded to amino acids in the lysosomes. In addition to non-specific protein degradation, cathepsins may play a role in specific processing of proteins (Quinn and Judah, 1978; Bond and Barrett, 1980), degradation of collagen, fibrinogen, and histones, in bone degradation (Otto and Schirameister, 1997) and protein breakdown in sepsis (Hummel et al., 1998). Studies with rat soleus muscle have shown that lysosomal proteolysis is involved in atrophy of denervated muscle (Tischler et al., 1990; Weinstein et al., 1997). This indicates that co-ordination of the various protease systems occur during periods of muscle atrophy.

Observation with different types of muscle wasting has shown that both lysosomal and Ca^{2+}-activated proteases contribute to muscle proteolysis. The Ub-proteasome-dependent process appears mainly responsible for the rapid loss of cell proteins, especially of myofibrillar components (Temparis et al., 1994; Mitch et al., 1994; Furono et al., 1990; Whipple and Koomaraie, 1991). Studies on different proteolytic pathways in L6 myotubes (Fernandez and Sainz, 1997) have indicated that degradation of sarcoplasmic and myofibrillar proteins are independently regulated. Both lysosomal and non-lysosomal proteolytic pathways are involved in regulation of total protein degradation in L6 myotubes. Similar investigation with C2C12 myotubes (Ebisui et al., 1995) demonstrated that both lysosomal and proteasome systems participated in the IL-6-induced degradation of long-lived intracellular proteins. These observations have shown protein turnover in muscle cells are mediated by a mixture of

proteolytic systems. However, it is unclear of which substrates are degraded by individual protease and how the proteases are regulated.

Protease Inhibitors

All endogenous cysteine protease inhibitors (cystatin superfamily) can inhibit calpains, lysosomal enzymes and the proteasome. Calpain is specifically inhibited by its endogenous inhibitor calpastatin. Calpastatin can prevent both enzymatic activation and the expression of calpain catalytic activity. Calpain and calpastatin are co-localized (Kumamoto et al., 1992) and calpastatin activity exceeds calpain activity in many cells (Kawasaki and Kawashima, 1996). Calpastatin was first cloned in 1987 (Emori et al., 1987) and its sequence is not homologous to any known polypeptide including the cystatins, which inhibit many of the cysteine proteases. Different calpastatin polypeptides can be produced by either alternative splicing, from different start sites of translation or from transcription of the calpastatin mRNA or gene (Cong et al., 1998; Lee et al., 1992b). Most calpastatin molecules comprise four repeating domains plus an N-terminal domain and domain L/XL (Figure 2). Each of repeated domains can bind one calpain molecule and possess inhibitory activity (Maki et al., 1987; Emori et al., 1987). Use of BIAcore instruments to examine the interaction site between calpastatin and calpain (Takano et al., 1995) has revealed that three sub-domains of the reactive site of calpastatin bind to Domain IV of the large calpain subunit, the active site and Domain IV' of the small subunit, respectively. Interestingly, the muscle-specific



Bovine calpastatin

Figure 2. Schematic diagram showing the domain structure of bovine cardiac calpastatin. A human erythrocyte calpastatin lacks domain XL, L and domain I (Imajoh et al., 1987). Rabbit and human skeletal muscle calpastatins do not have domain XL (Emori et al., 1987; Lee et al., 1992b).

calpain, p94, which does not associate with the small subunit (Kinbara et al., 1998), is not inhibited by calpastatin (Sorimachi et al., 1995).

As for synthetic inhibitors, a variety of peptidic, peptide mimetic and non-peptide inhibitors have been recently developed. Calpain inhibitors I and II, calpeptin, leupeptin, and E-64, a naturally occurring epoxysuccinate first isolated from fungi, and its derivatives (E-64c and E-64d) are widely used (Sorimachi et al., 1997; Gregory and Bihovsky, 1998; Hanada et al., 1978). Although these inhibitors efficiently inhibit both μ- and m-calpains, they are not very specific as they also inhibit other cysteine proteases and the proteasome as well (Figueiredo-Pereira et al., 1994; Tsubuki et al., 1996). PD 150606, a α-mercaptoacrylic acid derivative, is a relatively potent, selective and reversible calpain inhibitor developed by Wang and colleagues (Wang et al., 1996a). PD150606 has a distinct inhibitory mechanism. It binds to the Ca^{2+}-binding domain of calpain (Lin et al., 1997) and thereby interferes with calpastatin binding. Other inhibitors such as chloroquine, methylamine and NH_4Cl are commonly used in lysosome studies (Tischler et al., 1990; Baracos et al., 1995; Gordon and DeMoss, 1999; Myers et al., 1995; Minor et al., 1991).

Protein-Protein Interaction Studies and the Yeast Two-Hybrid System

Protein-protein interactions are intrinsic to virtually every cellular process. Protein-protein interactions have generally been studied using biochemical techniques such as cross-linking, co-immunoprecipitation, protein affinity chromatography, affinity blotting, and library-based methods i.e. protein probing, phage display and two-hybrid

system (Phizick and Fields, 1995). Functional protein-protein interactions using the yeast two-hybrid techniques have been detected for all levels of cellular biology (Estojak et al., 1995; Young 1998; Dress 1999) since it was developed in 1989 (Fields and Song, 1989). The yeast two-hybrid system takes advantage of the properties of the GAL4 protein of *Saccharomyces cerevisiae*. This protein is a transcriptional activator required for the expression of genes encoding enzymes for galactose utilization. The GAL4 protein consists of two separable and functionally essential domains: an N-terminal domain which binds to specific DNA sequence (UAS); and a C-terminal domain containing acidic regions, which is necessary to activate transcription. The system of two hybrid proteins contain portions of GAL4: the GAL4 DNA-binding domain fused to a protein 'X' and a GAL4 activating region fused to a protein 'Y'. If X and Y can form a protein-protein complex and reconstitute proximity of the two GAL4 domains, transcription of a gene regulated by UAS occurs (see Figure 3). Examples of protein partners that have been detected by this system include CDK6 and its inhibitor, p18 (Guan et al., 1994), c-jun/c-fos (Kato et al., 1992), p53/T-antigen (Li and Fields, 1993), the growth factor receptor-associated protein Grb2 and human Sos1 guanine nucleotide exchanger (Chardin et al., 1993), Ras and the protein kinase Raf (Vojtek et al., 1993), and protein phosphatase PP1α2 and Rb (Durfee et al., 1993).

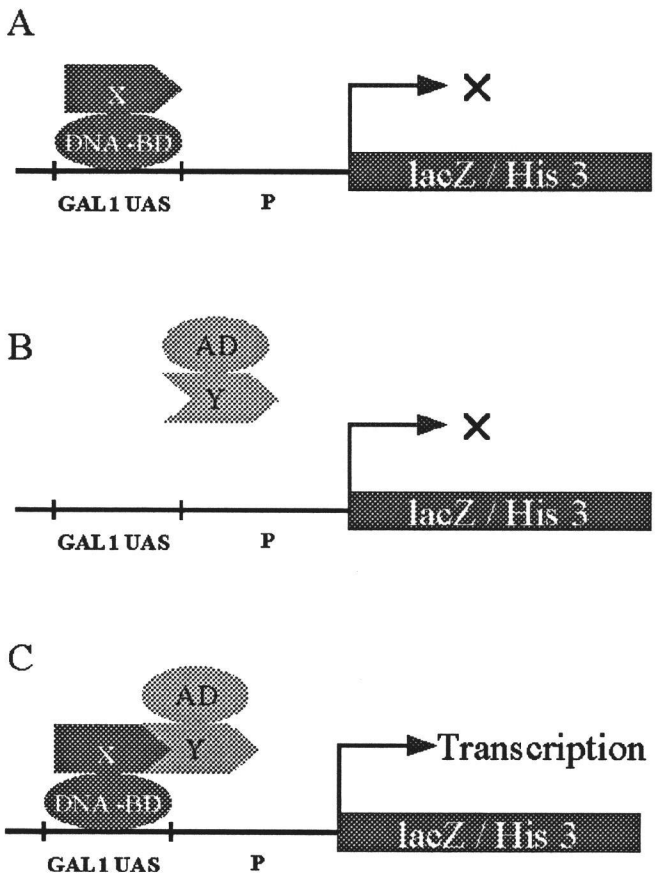


Figure 3. The two-hybrid system. A: The GAL4 DNA-binding domain hybrid does not activate transcription if protein X does not contain an activation domain. B: the GAL4 activation domain hybrid does not activate transcription because it does not localize to the DNA-binding site. C: Interaction between X and Y reconstitues GAL4 function and results in expression of the reporter gene.

MATERIALS AND METHODS

Coordination of Proteases in Control of Muscle Protein Degradation

Myoblast cultures

Rat myoblast L8 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Gaithersburg, MD) with addition of 1000 mg/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-100 μg/ml Streptomycin (Cellgro Mediatech, Herndon, VA) 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 myotube maintenance.

For long-term storage or subculture, adherent cells were detached from the plate by trypsinization with 2.5mg/ml trypsin (GibcoBRL, Gaithersburg, MD) and 3-min incubation at 37°C. FBS medium was immediately added to stop further trypsinization. Suspended 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 10:1 ratio of FBS medium and DMSO (Sigma, St. Louis, MO) and stored at -80°C or in liquid nitrogen.

Measurement of total protein degradation and stability of individual proteins

Calpastatin inhibitory domain-transfected myoblast L8 cells (L8/CID cells; Huang and Forsberg, 1998) were cultured in 5-cm culture dishes (Corning, NY) in the medium containing hygromycin B (200 μg/ml; GibcoBRL) and geneticin (400 μg/ml; GibcoBRL) to maintain the existence of the transgenes. Total protein degradation was estimated from the rate of production of free tyrosine from pre-labeled muscle proteins (pulse-chase experiment; Goldberg and St. John, 1976). Each treatment was completed in triplicate. At 90-100% differentiation (day 6), L8/CID cells were cultured for 24 hr in the presence of 0.33 μCi/ml of [^{3}H] tyrosine (42.3 Ci/mmol; NEN, Boston, MA) with and without isopropyl β-D-thiogalacto-pyranoside (IPTG; CID inducer, Sigma, St. Louis, MO) or methylamine (5-20 mM; lysosomal protease inhibitor, Sigma) or Z-Leu-Leu-Leu-H (aldehyde) (ZLLL, 15-100 nM; proteasome inhibitor, Peptide Institute, Osaka, Japan) or combination of the inhibitors. Following this, three plates of L8/CID myotubes were taken and processed for determination of the initial levels of radioactivity associated with protein fractions. In the remaining plates, the radioactive media were removed and the cultures were washed twice and refilled with DMEM containing 2 mM tyrosine (chase; Sigma). At this time, 1.5 ml of medium were taken from each plate and radioactivity was measured to determine the radioactivity present at time zero. The plates were cultured for an additional 6, 12 or 24 hr. At the end of incubation, 1.5 ml of medium were taken from each plate, treated with 10% TCA (final w/v) and precipitated by centrifugation at 14000xg for 10 min. The supernatants were then measured for radioactivity. From these measurements, total protein degradation

(D) and the percentage reduction of protein degradation (R) caused by IPTG and inhibitors were calculated as follows: D = radioactivity at time 6, 12 or 24 hr - radioactivity at time 0 and $R = 100 \times D - D_{inhibitor} / D$. Stabilities of individual proteins (eg. α-actinin, filamin, desmin, m-calpain, tropomyosin and dystrophin) were assessed using Western blot analysis.

Preparation of muscle myofibrillar- and non-myofibrillar protein-rich fractions

The muscle samples were fractionated into sarcoplasmic and myofibrillar fractions. To accomplish this, muscle samples were washed twice with phosphate-buffered saline (PBS) and were scraped from the culture dishes and resuspended in 1 ml of low salt buffer (40 mM NaCl, 1 mM dithiothreitol, 0.1 mM EGTA, 0.1% Triton X-100 in 5 mM sodium phosphate, pH 6.8). Cells were then homogenized using a Dounce homogenizer with 15 strokes of a tight fitting pestle. The homogenate was centrifuged at 1000xg for 10 min. The pellet containing myofibrillar proteins was dissolved in 1 ml of 0.5 N NaOH with 0.1% Triton X-100. The supernatant contained sarcoplasmic (non-myofibrillar) proteins. To this 10% TCA (final w/v) was added and the mixture was chilled at 4°C for 1 hr. After centrifugation at 14000xg for 10 min, the pellet was dissolved in 1 ml of 0.5 N NaOH with 0.1% Triton X-100. Nine ml of EcoLume Liquid Scintillation Cocktail (ICN, Costa Mesa, CA) was mixed with the protein fractions. The radioactivity in the protein fractions was then counted in liquid scintillation counter.

Preparation of protein extracts from cultured cells

Cell protein was recovered according to Wang et al. (1996b). Following treatments, cells were washed twice with PBS and lysed at room temperature for 5 min by adding 500 µl of SDS lysis/protease inhibitor solution (20 mM Tris-Cl pH 7.4, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 2% SDS, 0.5 mM PMSF (Boehringer Mannheim, Indianapolis, IN), 10 µg/ml 4-(2-aminoethyl)-benzene-sulfonylfluoride (AEBSF) hyhrochloride (Sigma), 5 µg/ml leupeptin (Sigma), 10 µg/ml N-tosyl-L-phenylalanine chloromethyl ketone (TLCK; Sigma), 10 µg/ml TPCK (Sigma), 10 µg/ml E-64 (Peptide International, Louisville, KY) and 0.37 mg/ml aprotinin (Sigma). One hundred µl of 100% TCA were added to the lysate and the DNA aggregate was removed. The lysate was then centrifuged at 3600xg for 5 min. Pellets were washed with 1 ml of 2.5% TCA (w/v), neutralized and dissolved in 25-50 µl of 3 M Tris base. Protein concentration in the lysate was determined by the method of Lowry et al. (1951). Aliquots of protein lysate were separated by SDS-PAGE and analyzed by immunoblotting.

SDS-PAGE and Western blots

Proteins were subjected to SDS-PAGE (6.5-10%) according to Laemmli (1970). The gel was run at 120V until the bromophenol blue dye front reached the bottom of the gel. After electrophoresis was completed, the gel was either stained with Coomassie blue or transferred to the electroblotter apparatus (BioRad, Hercules, CA). The proteins were electrotransferred to Optitran nitrocellulose membranes (Schleicher & Schuell,

Keene, NH) overnight at 30V, followed by 2 hr at 60V. Non-specific sites on the membrane were blocked with 5% non-fat dry milk and 1% BSA in TBST (20 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.1% v/v Tween 20) for 1 hr. The membrane was then incubated for 2 hr with monoclonal antibody against α-actinin (1:3500, Sigma), filamin (1:1500, Sigma), desmin (1:1000, Sigma), dystrophin (1:2000, Sigma), tropomyosin (1:1000, Sigma), lac I (1:1000; polyclonal, Stratagene, La Jolla, CA) and m-calpain (1:850; rabbit anti-rat polyclonal prepared in our laboratory). The bound antibody was detected by 1-hr incubation with conjugated secondary antibody. Detection of bands was carried out with the ECL immunoblotting detection system (ECL, Amersham Pharmacia, Piscataway, NJ). Blots were quantified by scanning densitometry. The Object Average method of Image QuaNT, Molecular Dynamics software, was used to determine the amount of each image based on the area and intensity of the blot.

Cell Toxicity assay

To examine if inhibitors and IPTG were toxic to the cells. Toxicity of cultured cells was assayed by using Cytotoxicity assay kit (Promega, Madison, WI). In this assay, amounts of lactate dehydrogenase (LDH) which leaked into the medium were detected. Briefly, 50 μl of cell culture medium was incubated with 50 μl of substrate mix for 30 min in the dark. The reaction was stopped by adding 50 μl of stop solution. OD_{490} of treated sample was compared to that of untreated sample to assess cell toxicity.

Statistical analysis

In the studies of total protein degradation, each experiment was repeated twice for ZLLL and methylamine treatments and at least three times for IPTG treatment. Triplicate measurements were made for each experiment. The data were analyzed using multifactor ANOVA for the effects of different experiments. *Scheffe*'s statistic was used to test the contrasts, the comparison of each concentration to control (no inhibitor). The experiments with small numbers of contrasts were tested by comparison to Bonferroni t statistic.

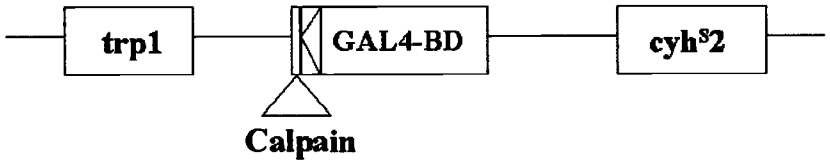
For the experiments with the combined treatments, each experiment was repeated three times using three replicates for each treatment. The experiment effects and comparison of each treatment to control (no inhibitor at 24 hr after serum withdrawal) were assessed using multifactor ANOVA and *Scheffe*'s statistic, respectively. Paired t-test was used to test the difference of control at 0 hr and 24 hr after serum withdrawal. A level of significance of 5% was adopted for all comparisons and the SAS system for windows was used as the statistical software.

Identification of Calpain Substrates Using the Yeast Two-Hybrid System

Plasmid construction

Construction of dominant negative m-calpain: Full length human μ- and m-calpain cDNA, cloned into expression plasmid, pAS2-1 (pASμCL and pASmCL, respectively; Figure 4), was kindly provided by Hiroyuki Sorimachi (University of Tokyo, Japan).

DNA-BD vector : pAS2-1



DNA-AD vector : pACT2, pGAD10

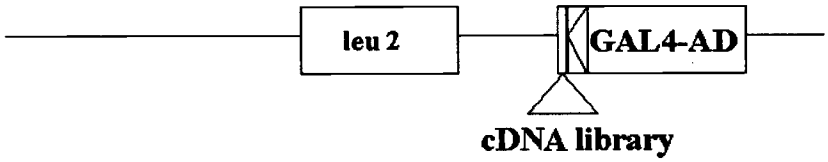


Figure 4. Expression vectors in the yeast two-hybrid system. The diagrams show genes necessary for selection and screening in the yeast two-hybrid system. Calpain cDNAs were subcloned into pAS2-1, fused in-frame to GAL4-DB. Library cDNAs were inserted undirectionally into pGAD10's EcoRI site.

Active site mutagenesis of m-calpain was conducted by polymerase chain reaction (PCR) using pASmCL as a template with a forward primer FPC105A (5'-AGG AGC CCT AGG TGA CGC CTG GCT G-3') and a reverse primer RPhm808 (5'-GGC TCC GGT GAC CGA GTA CGC GTG -3'). The optimal conditions for PCR reaction consisted of 2 mM $MgCl_2$, 0.2 mM dNTPmix, 10 µl Taq DNA polymerase buffer, 2 µl (20 ng) DNA template, 1 µl (50 pmol) forward primer, 1 µl (50 pmol) reverse primer, 0.5 µl Tag DNA polymerase (5 u/µl, Promega, Madison, WI), and water to make 100 µl. To generate active site-mutated DNA fragments, PCR was performed for 35 cycles of 94°C for 1 min, 66°C for 50 sec, and 72°C for 1 min. The *AvrII-BstEII*-cut PCR fragmentes were then inserted into pASmCL (a plasmid carrying GAL4-BD-hm-calpain fusion cDNA) cleaved with *AvrII* and *BstEII* to create pASmCLDN. Correct orientation and composition of the insertion were confirmed by DNA sequencing. All restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs, (Beverly, MA).

Construction of autolytic forms of m-calpain (78DN): The 78K autolysed form (Nishimura and Goll, 1991) of dominant negative m-calpain (mCLDN) was generated from pASmCLDN by PCR with *NcoI*-containing forward primer 78khmfp (5'-TTA TCC ATG GAA TCC CAC GAG AGG GCC ATC AAG TA-3') and *SalI*-containing reverse primer pashmrp (5'-TGC AGG TCG ACG GAT CAA TCA GAG AT-3'). The reaction for generating the 78K fragments was accomplished under 35 cycles of 94°C for 1 min, 58°C for 50 sec and 72°C for 2.30 min. The 78K DNA fragment was then subcloned into pAS2-1 (Figure 4; Clontech Laboratories, Palo Alto, CA) to create

pAS78DN. Correct in-frame and orientation of the inserts were confirmed by DNA sequencing.

Construction of domain IV-deleted 78K m-calpain (78Δ4): The forward primer 78khmfp and *SalI*-containing reverse primer pasΔ4rp (5'-GAG TCG TCG ACC TCC ATC ATC AAT GTC ATC CTC G-3') were used to amplify 78Δ4 fragments from pAS78DN plasmid. The PCR reaction was accomplished under 35 cycles of 94°C for 1 min, 60°C for 50 sec and 72°C for 2 min. DNA fragment 78Δ4 was then subcloned into pAS2-1 (Clontech, Palo Alto, CA) to create pAS78Δ4. Correct in-frame and orientation of the inserts were confirmed by DNA sequencing.

Preparation of library plasmid DNA

A pre-made human skeletal muscle cDNA plasmid library (Figure 4; Clontech, Palo Alto, CA) was amplified on solid medium to minimize uneven amplification of the individual clones. Briefly, the *E. coli* library transformants were plated directly onto LB/amp plates at high density (20,000 colonies/150-mm plate). Following overnight incubation at 37°C, the colonies were scraped into 2L of LB/amp broth and were incubated at 37°C for 3 hr with shaking. The cells were collected by centrifugation. CsCl gradient purification was used to isolate the plasmid libraries (Heilig et al., 1994)

Preparation of yeast competent cells for library transformation

The modification of lithium acetate (LiAc) method developed by Ito et al. (1983) was used for preparing yeast competent cells. *S. cerevisiae* strain Y190 (MAT_a,

his$^-$, *trp*$^-$, *leu*$^-$) or Y187 (MAT$_\alpha$, *his*$^-$, *trp*$^-$, *leu*$^-$) were inoculated into YPD or appropriate selective media, vortexed to disperse any clumps, and transferred into 150 ml of YPD. The culture media were incubated at 30°C for 16-18 hr with shaking at 250 rpm. Overnight culture was transferred to 1L of YPD to produce an OD_{600} = 0.2-0.3, incubated at 30°C for 3 hr with shaking at 230 rpm. The cell pellet was collected by centrifugation at 1000xg for 5 min, washed once in water, and resuspended in 8 ml of freshly made TE/0.1M LiAc (1x) solution (Sigma).

Yeast transformation

The LiAc-mediated method modified by Schiestl and Gietz (1989), Hill et al. (1991), and Gietz et al. (1992) was used to introduce DNA into yeast. In the DNA library transformation, sequential transformation was preferred to simultaneous transformation because it typically resulted in transformation efficiencies of 10^5 per μg of DNA when using a single type of plasmid and it used less plasmid DNA. Shortly, 8 ml of yeast competent cells in TE/LiAc solution were mixed with 0.75-1 mg of the library plasmid (0.5-0.75 mg library plasmid +1 mg bait plasmid for co-transformation) and 10 mg of herring testes carrier DNA (20 mg for co-transformation). Polyethylene glycol (PEG)/LiAc solution (40% PEG, Sigma: 1xTE: 1xLiAc, Sigma) was then added and the mixture was incubated at 30°C with shaking. After the incubations, DMSO (Sigma) was added to 10% (v/v) and mixed gently by inversion. The cells were heat-shocked for 15 min in a 42°C water bath and placed on ice for 5 min. The pelleted cells were collected by centrifugation for 5 min at 1000xg. The cells were resuspended in 10

ml of TE buffer (pH 7.5) and were then plated on the synthetic dropout (SD) medium (SD/-leu,-trp,-his,+3AT) to select for transformants containing the plasmid(s) that expressed β-galactosidase and histidine-3 genes (Figure 5). The transformation efficiency was determined by the number of colonies growing on SD medium that selected for only one of the plasmids (e.g. SD/-leu medium for library plasmid and SD/-trp medium for bait plasmid). The co-transformation efficiency was determined by the number of colonies growing on SD/-leu,-trp medium that selected for both plasmids.

Cycloheximide selection

Cycloheximide was used to select for colonies that had lost the DNA-BD/bait plasmid (Guthrie and Fink, 1991). A colony of yeast cells carrying two different plasmids (i.e. the DNA-BD/calpain plasmid and the AD/library plasmid) was resuspended in 200 µl of water and 10-100 µl of the cell suspension were spread on SD/-leu medium containing 10 µg/ml cycloheximide. The plates were incubated at 30°C for 3-5 days until individual Cyh^R colonies appeared. The DNA-BD/calpain plasmid that contained the wild-type *CYH*^{S}2 gene would render yeast host strains Y190 to become sensitive to cycloheximide. Therefore, the AD/library plasmid would be retained while the DNA-BD/calpain plasmid had spontaneously been lost from the cell. The loss of pAS2-1 plasmids was verified by transferring the Cyh^R colonies to SD/-trp plates. Colonies that had lost pAS2-1 plasmids would not grow.

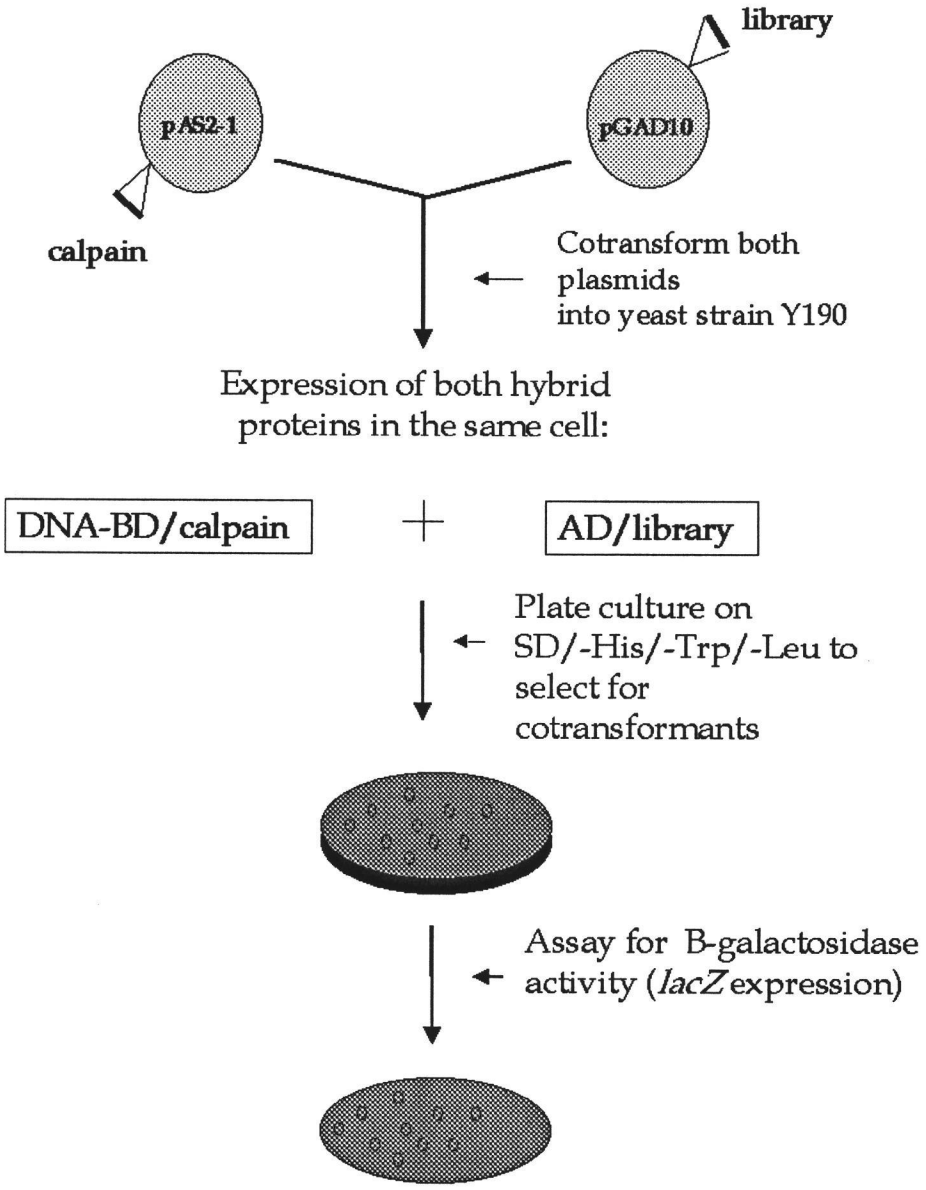


Figure 5. Schematic diagram of using the yeast two-hybrid system to screen cDNA library for an interaction between two proteins.

Yeast mating

A haploid state of yeast strain Y190 and Y187 which differed physiologically in mating types can reproduce sexually by cellular and nuclear fusion to produce a diploid organism. Therefore, it was used to verify that the candidate AD/library proteins identified in the two-hybrid library screen could activate the reporter genes only in the presence of the DNA-BD/target protein. In the mating procedure, various control plasmids and candidate plasmids to be tested were transformed into proper hosts and the mating pairs were set up as listed in Table 1. One colony of each type was placed in a 1.5-ml microcentrifuge tube containing 0.5 ml of YPD medium. The medium was vortexed and incubated at 30°C with shaking at 250 rpm for 8 hr. The mating culture was then spread on SD/-trp,-leu and SD/-trp,-leu,-his,+3-AT plates. The plates were incubated at 30°C for 4-5 days or until diploid cells appeared.

Table 1. Yeast mating partners to eliminate false positives

Plasmid 1 in Y 187 (*MAT*α)	Plasmid 2 in Y 190 (*MAT*a)	LacZ phenotype for a true positive	His3 phenotype for a true positive
pAS2-1 (DNA-BD)	pACT2 (AD)	white	no growth
pAS2-1/calpain (DNA-BD/calpain)	pACT2	white	no growth
pAS2-1	pGAD10/library (AD/target)	white	no growth
pAS2-1/calapin	pGAD10/library	blue	positive growth
pLAM5' (DNA-BD/control)	pACT2/library	white	no growth

β-Galactosidase Assays

Colony-lift filter assay: The filter assay was primarily used to screen large numbers of transformants that survived the *His*3 growth selection in a GAL4 two-hybrid library screening and also was used to assay for interactions between two known proteins. A filter was placed over the surface of the plate of fresh colonies to be assayed (grown at 30°C for 2-4 days), then carefully lifted off the agar plate and was placed in a -80°C freezer with the colonies facing up for at least 1 hr. The filter was allowed to thaw at room temperature. This freeze-thaw cycle lyses yeast cell walls and permeabilizes the cells. The filter was then placed, colony side up, on presoaked filters (in Z buffer/X-Gal solution; Sigma) and incubated at room temperature until the appearance of blue colonies. The blue color indicates the expression of *lac Z* reporter gene.

Culture assay using ONPG as substrate: One colony of each *lac* Z-positived and control yeast were cultured overnight in 5 ml YPD medium. Two ml of the overnight cultures were transferred to 8 ml YPD medium and the fresh culture was incubated at 30°C with shaking until OD_{600} of 1 ml cultures reached 0.5-0.8. The cells from 1.5 ml cultures were washed twice with 1.5 ml of Z buffer and, after the last wash, the pelleted cells were resuspended in 300 μl of Z buffer. One hundred μl of the cell suspension was transferred to a new microcentrifuge tube. Cells were then frozen in liquid nitrogen and thawed in 37°C water-bath for 1 min. The freeze/thaw cycle was repeated three more times. β-galactosidase activity in the cell lysate was measured by adding 0.7 ml of Z buffer/β-mercaptoehtanol (0.27% v/v) and 160 μl of ONPG (4 mg/ml; Sigma) in Z buffer. After the yellow color was developed, 0.4 ml Na_2CO_3 was added. The reaction

tube was then centrifuged at 14000xg for 10 min. Absorbance of the supernatant was read at OD_{420} against a blank in which 100 µl of Z buffer replaced the cell suspension in the reaction. β-galactosidase units were calculated by 1000x OD_{420} /(TxVxOD_{600}); where "T" was elapsed time in minutes (from ONPG addition to color development) and "V" was 0.1 x the concentration factor (= 5).

Protein extraction of yeast culture for Western blot

Five ml of overnight culture in SD selection medium of transformed yeast strain was prepared from a single isolated colony. The cultures were vortexed and inoculated into 50 ml of YPD medium. After incubation at 30°C with shaking until the OD_{600} had reached 0.4-0.6, the cultures were quickly chilled and centrifuged at 1000xg for 5 min at 4°C. Cell pellets were washed in 50 ml of ice-cold water. The pellets recovered by centrifugation were resuspened in cracking buffer/inhibitor solution. The cracking buffer (7.08 M urea, 4.4% w/v SDS, 35.4 mM Tris-HCl pH 6.8, 0.09 mM EDTA, 0.35 mg/ml bromophenol blue, 0.88% v/v β-mercaptoethanol) contained protease inhibitors (0.006 mg/ml pepstatin A; Sigma, 0.002 mM leupeptin; Sigma, 9 mM benzamidine; Sigma, 0.023 mg/ml aprotinin; Sigma, 0.05 mM PMSF; Boehringer Mannheim) and was prepared immediately before needed and pre-warmed to 60°C. The ratio of 100 µl of cracking buffer to 7.5 OD_{600} units of cells was used. Total number of OD_{600} units was equal to OD_{600} of 1 ml sample x the culture volume. Proper amount of cracking buffer was added to cell pellets. The cell suspension was then transferred to a crew-cap microcentrifuge tube containing 80 µl of glass beads per 7.5 OD_{600} units of cells. The

sample was heated at 70°C for 10 min and then vortexed for 1 min. The supernatants were collected by centrifugation at 14000 rpm for 5 min in 4°C. The remaining pellet debris was placed in a boiling water bath for 5 min, vortexed for 1 min and centrifuged to collect the supernatants. Supernatants were combined and stored in -70°C freezer.

SDS-PAGE and Western blot

Proteins were subjected to 7.5-10% SDS-PAGE according to Laemmli (1970). The gel was run at 120V until the bromophenol blue dye front reached the bottom of the gel. After electrophoresis was completed, the gel was either stained with Coomassie blue or transferred to the electroblotter apparatus (BioRad, Hercules, CA). The proteins were electrotransferred to Optitran nitrocellulose membranes (Schleicher & Schuell, Keene, NH) for overnight at 30V, followed by 2 hr at 60V. Non-specific sites on the membrane were blocked with 5% nonfat dry milk and 1% BSA in TBST (20 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.1% v/v Tween 20) for 1 hr. The membrane was then incubated for 2 hr with monoclonal antibody against GAL4-DB (1:5000, Clontech) or GAL4-AD (1:5000, Clontech). The bound antibody was detected by 1-hr incubation with conjugated secondary antibody. Detection of bands was carried-out with the ECL immunoblotting detection system (ECL, Amersham Pharmacia, Piscataway, NJ).

Proteolysis of phosphorylase-a and creatine kinase

Twenty μg rabbit muscle glycogen phosphorylase and creatine kinase (Sigma; P-1261, C-3755) were incubated for 15-30 min at 30°C in buffer A (50 mM Tris-HCl

pH 7.4, 0.2 mM EGTA, 0.5 mM Dithiothreitol) in the presence of 2 mM $CaCl_2$ with or without 0.5 units of m-calpain (Sigma; P-4533). The reaction was then stopped by the addition of 10 mM EDTA (final concentation). After the reaction had stopped, the reaction mixtures were subjected to SDS-PAGE and the gels were then stained with Coomassie blue.

RESULTS

The expression of *CID* in L8/CID cell lines used in the experiments is under-control of the LacSwitch expression system. Therefore, CID over-expression is achieved by addition of IPTG to culture media. IPTG inhibits Lac I binding to the Lac promoter, and thereby allows *CID* to be expressed. The presence of IPTG does not affect Lac I and m-calpain mRNA (Huang and Forsberg, 1998). In this cell line, CID fragments are expected to non-selectively inhibit both μ- and m-calpains in an unregulated manner.

Earlier studies with L8 cells (Hong and Forsberg, 1994) have documented that serum withdrawal caused an approximately 20% increase in total protein degradation. Therefore, the accelerated proteolysis of L8 cultured muscle cells with the use of various protease inhibitors provides a good system for studying the protein degradation pathways responsible for protein breakdown and the loss of different muscle proteins.

The continuous turnover of cell protein is typically measured in cells using isotopic amino acid tracers in "pulse-chase" experiments (Goldberg and St. John, 1976). After a short labeling of proteins, the decay of radioactive polypeptides is followed over time. To dilute the reincorporation of radioactive amino acids released by proteolysis, cells are administered large amounts of non-radioactive amino acids, or protein synthesis is blocked with antibiotic inhibitors (i.e. cyclohexamide).

Effects of IPTG on Total Protein Degradation in L8-CID muscle cells

Total protein degradation was estimated by measuring free ^{3}H-tyrosine released into the media following serum deprivation. Because muscle can neither synthesize nor degrade this amino acid, tyrosine release reflects the breakdown of proteins (Taillandier et al., 1996). Rates of total protein degradation increased asymptotically during the first 30 hr after serum withdrawal (Figure 6). According to a preliminary study, IPTG at varying doses (0, 5, 7, 10 and 15 mM) were added to differentiated L8/CID cells. IPTG did not affect total protein degradation in both 6 and 12 hr following serum withdrawal (Figures 7 and 8). IPTG caused an approximate 20% reduction in degradation when 5-7 mM IPTG were added for 24 hr (Figure 9). However, those effects were not statistically significant (*Scheffe* test). Concentrations above 7 mM were toxic and caused cells to detach and die. Small inhibitory effects of CID expression on total protein degradation were unexpected since it was reported to reduce total protein degradation by approximately 60% when cells were exposed to IPTG for 24 hr (Huang and Forsberg, 1998). We also detected a similar degree of inhibition by IPTG in three other L8/CID clones where we varied the cell-differentiated period prior to administration of treatments (i.e. treatments were started at days 6, 8, or 10 after differentiation was induced; data not shown). Therefore, different clones and stages of differentiation did not contribute to the low effect of CID expression in this study. However, it appears, expression of CID did affect other aspects of muscle cell biology. Specifically, the m-calpain concentration assessed by Western blot (Figure 10) was upregulated by CID expression.

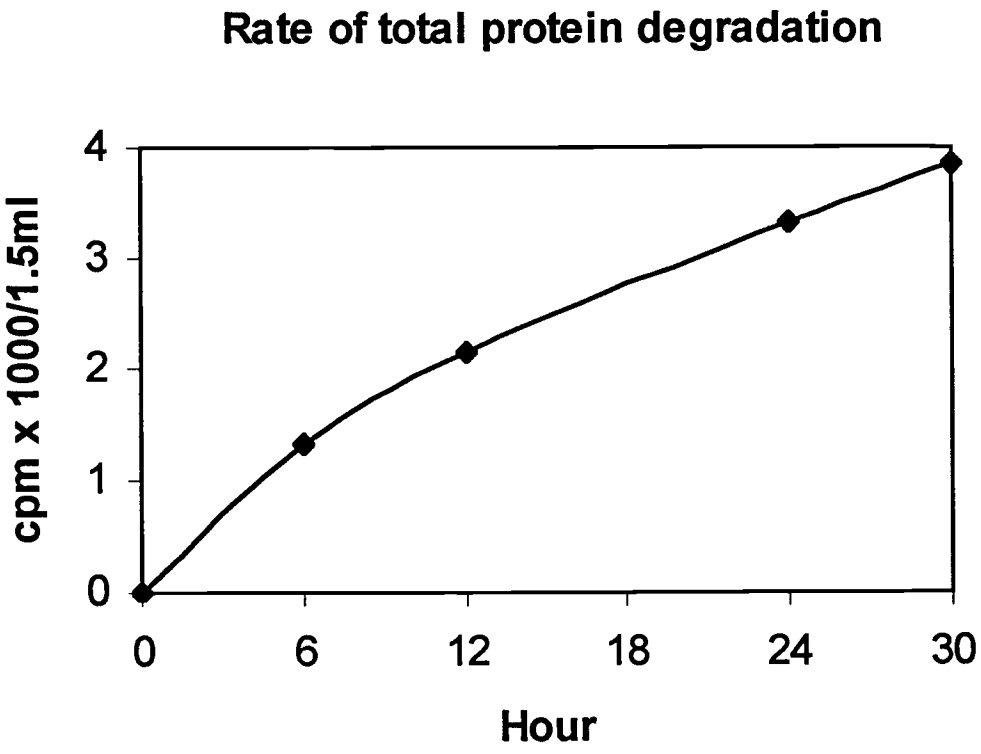


Figure 6. Time-dependent response of total protein degradation following serum withdrawal in L8/CID myotubes. ^{3}H-tyrosine-prelabeled L8/CID myotubes were incubated for different times (0, 6, 12, 18, 24 and 30 hr) in DMEM as described in text. After incubations, 1.5 mL-cultured media were collected and radioactivity was determined.

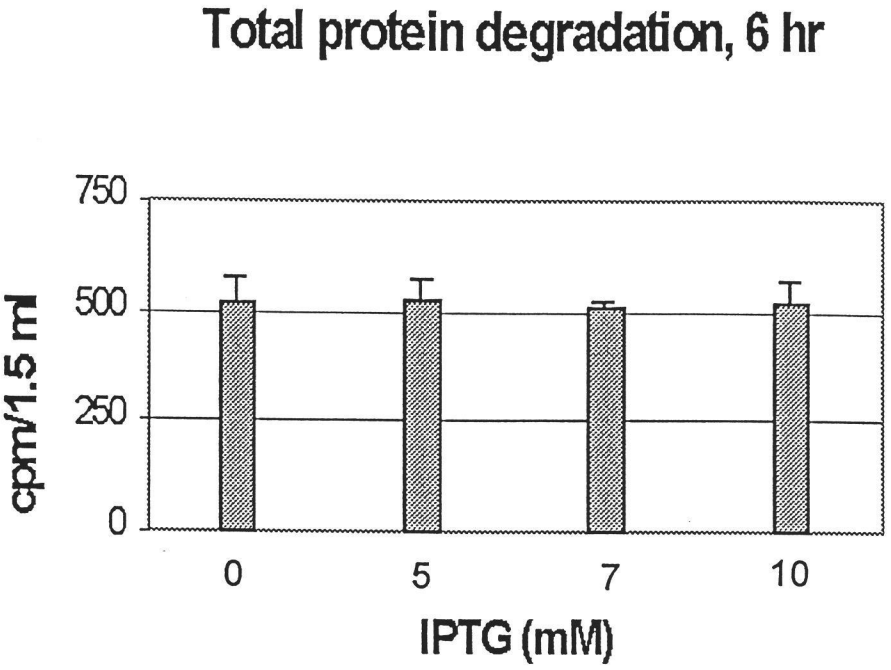


Figure 7. Effect of IPTG on total protein degradation at 6 hours following serum deprivation in L8/CID myotubes. ^{3}H-tyrosine-prelabeled L8/CID myotubes were incubated with different IPTG concentrations (0, 5, 7, 10 mM) in DMEM for 6 hours as described in text. After treatments, 1.5 mL-cultured media were collected and radioactivity was determined. Overall protein degradation of IPTG-treated cells did not differ significantly from control (0 mM IPTG). Results represent the mean of two repeated experiments. Triplicates were done in each experiment.

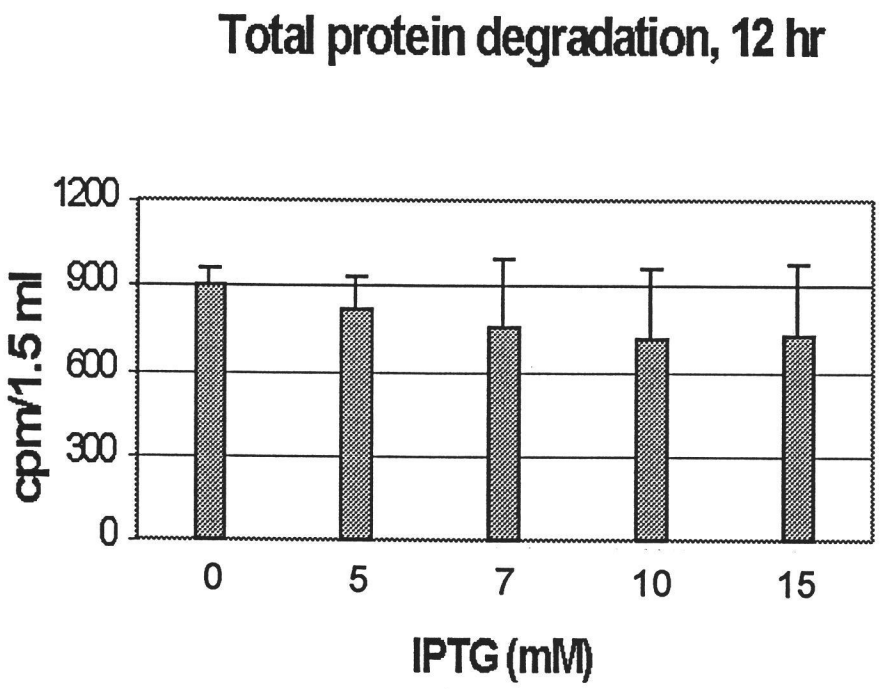


Figure 8. Effect of IPTG on total protein degradation at 12 hours following serum deprivation in L8/CID myotubes. ^{3}H-tyrosine-prelabeled L8/CID myotubes were incubated with different IPTG concentrations (0, 5, 7, 10, 15 mM) in DMEM for 12 hours as described in text. After treatments, 1.5 mL-cultured media were collected and radioactivity was determined. Overall protein degradation of IPTG-treated cells did not differ significantly from control (0 mM IPTG). IPTG concentrations above 7 mM were toxic to the cells. Results represent the mean of three repeated experiments. Triplicates were done in each experiment.

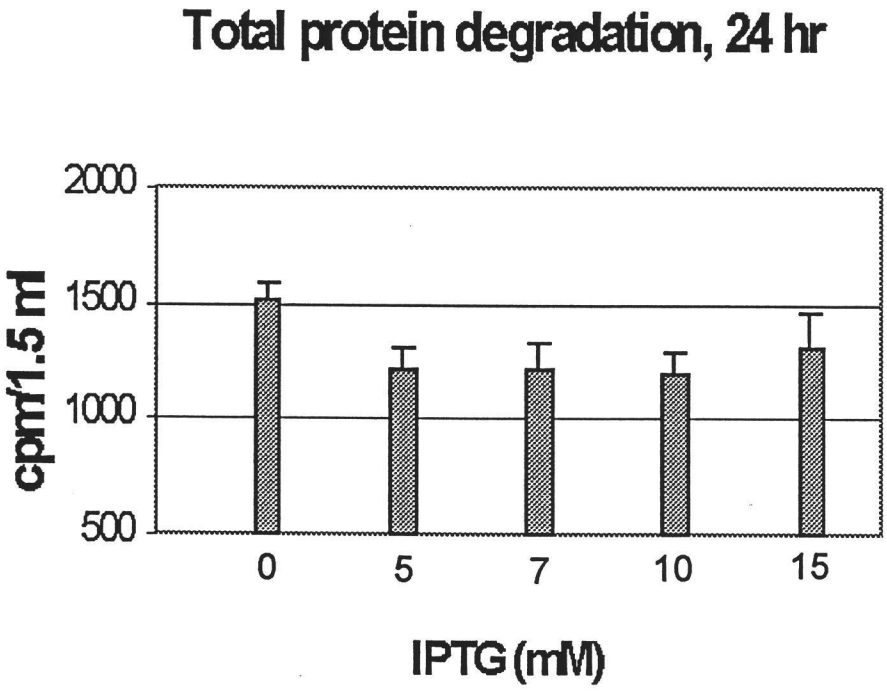


Figure 9. Effect of IPTG on total protein degradation at 24 hours following serum deprivation in L8/CID myotubes. ^{3}H-tyrosine-prelabeled L8/CID myotubes were incubated with different IPTG concentrations (0, 5, 7, 10, 15 mM) in DMEM for 24 hours as described in text. After treatments, 1.5 mL-cultured media were collected and radioactivity was determined. Overall protein degradation of IPTG-treated cells did not differ significantly from control (0 mM IPTG). At concentration 7 mM and higher, IPTG was toxic to the cells. Results represent the mean of three repeated experiments. Triplicates were done in each experiment.

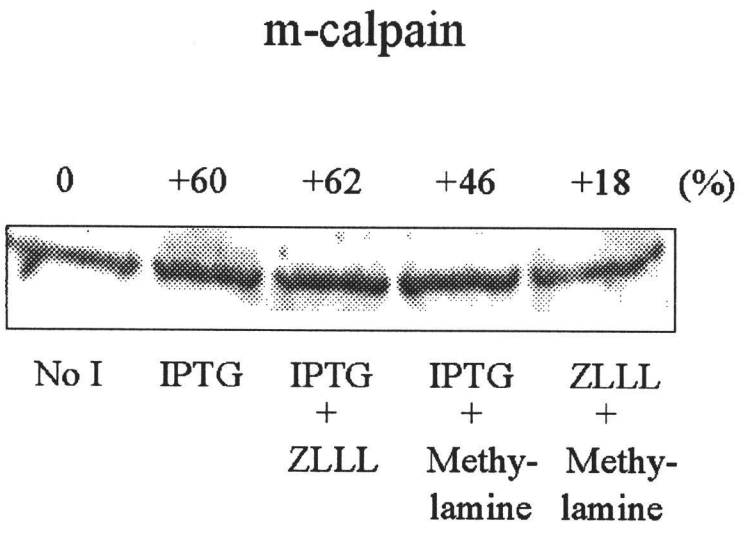


Figure 10. Effect of over-expressed CID, proteasome and lysosomal inhibitors on m-calpain concentration in L8/CID myotubes. L8/CID cell lines were treated with IPTG, ZLLL and methylamine for 48 hr. Forty μg of each cultured cell lysate protein was assessed by western blot. Polyclonal rat m-calpain antibody was used to probe the blot as described in the text. Numbers on top of the blot indicate percentage difference of each band's intensity compared to control (no inhibitor).

Effects of Methylamine on Total Protein Degradation

Methylamine is an agent that inhibits lysosomal enzymes by altering the pH of the lysosome. Methylamine was reported to cause major increases in lysosomal pH by 0.5 – 2.0 pH units. In addition, it causes changes in lysosome morphology (Myers et al., 1995). We evaluated effects of methylamine on protein degradation in cultured muscle cells in order to determine the contribution of the lysosome to total protein degradation (Figures 11 and 12). Methylamine significantly reduced total protein degradation in both 12 and 24 hr following serum withdrawal ($p<0.0001$). Ten-mM methylamine caused a 40% reduction in degradation. Concentrations above this were toxic to the muscle cells.

Effects of ZLLL on Total Protein Degradation

To evaluate the role that the proteasome plays in turnover of proteins in cultured muscle cells, we examined effects of proteasome inhibitors on release of radioactivity from pre-labelled myotubes. ZLLL or MG-132, a synthetic membrane-permeable proteasome inhibitor, effectively blocked ATP-dependent protease activity in CHO cell lines (Jensen et al., 1995; Nawaz et al., 1999). The effect of ZLLL was tested in the dose range of 30 to 100 nM. We found that ZLLL could significantly reduce total protein degradation in L8/CID cells at both 12 and 24 hr ($p<0.0001$). ZLLL caused a progressive reduction in total protein degradation and, at 30 and 50nM, inhibited total protein degradation by approximately 50 and 62%, respectively (Figures 13 and 14). Concentrations above this were toxic. At 30 nM, the effect of ZLLL on proteolysis

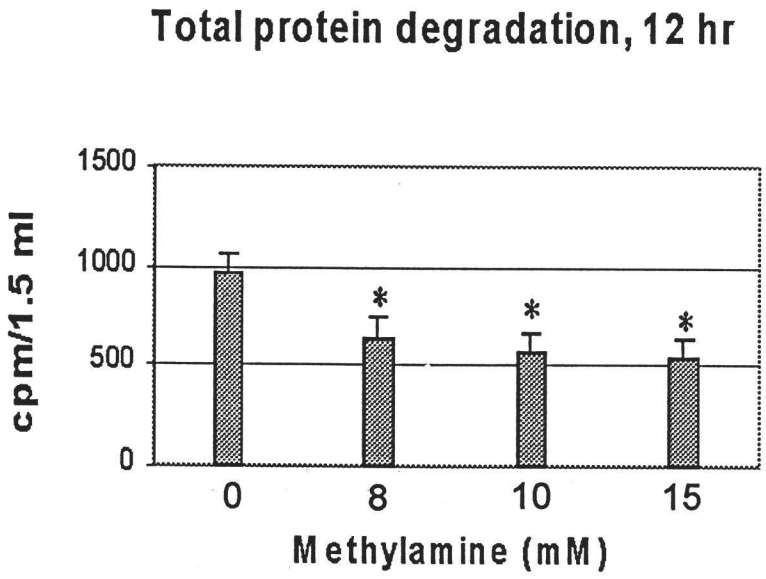


Figure 11. Effect of methylamine on total protein degradation at 12 hours following serum deprivation in L8/CID myotubes. ^{3}H-tyrosine-prelabeled L8/CID myotubes were incubated with different methylamine concentrations (0, 8, 10, 15 mM) in DMEM for 12 hours as described in text. After treatments, 1.5 mL-cultured media were collected and radioactivity was determined. " * " indicates this treatment differed significantly ($p<0.0001$) from control (0 mM methylamine). Methylamine at 15 mM was toxic to the cells. Results represent the mean of two repeated experiments. Triplicates were done in each experiment.

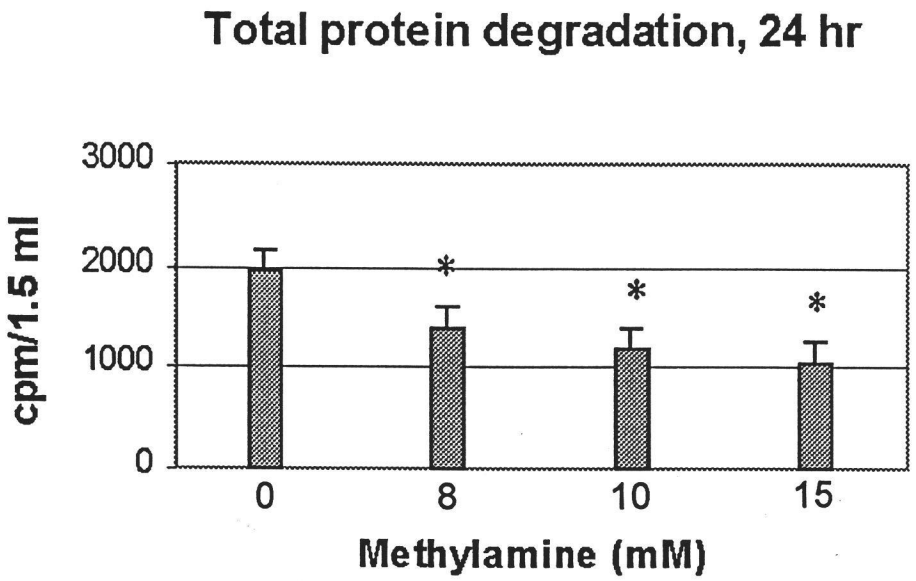


Figure 12. Effect of methylamine on total protein degradation at 24 hours following serum deprivation in L8/CID myotubes. ^{3}H-tyrosine-prelabeled L8/CID myotubes were incubated with different methylamine concentrations (0, 8, 10, 15 mM) in DMEM for 24 hours as described in text. After treatments, 1.5 mL-cultured media were collected and radioactivity was determined. " * " indicates this treatment differed significantly ($p<0.0001$) from control (0 mM methylamine). Methylamine at 15 mM was toxic to the cells. Results represent the mean of two repeated experiments. Triplicates were done in each experiment.

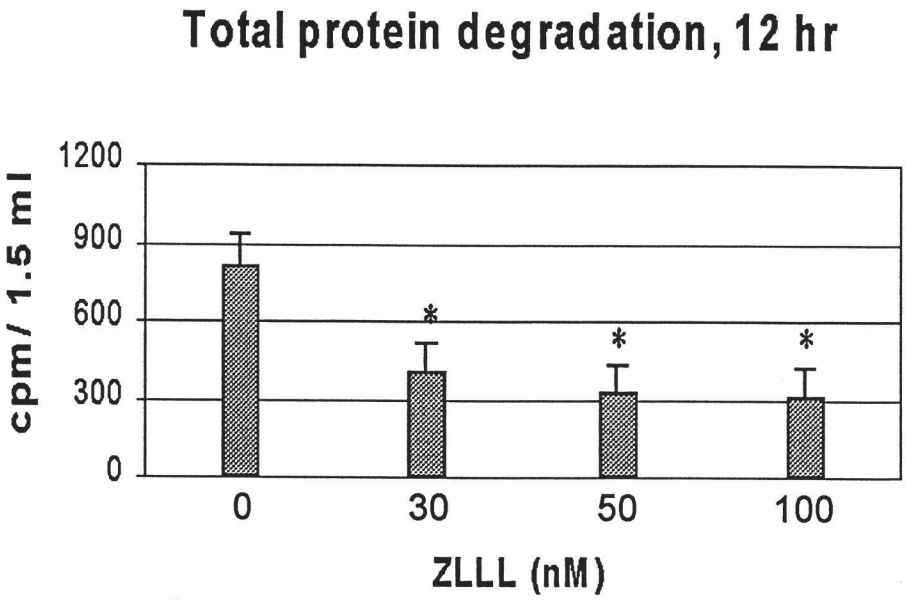


Figure 13. Effect of ZLLL on total protein degradation at 12 hours following serum deprivation in L8/CID myotubes. ^{3}H-tyrosine-prelabeled L8/CID myotubes were incubated with different ZLLL concentrations (0, 30, 50, 100 nM) in DMEM for 12 hours as described in text. After treatments, 1.5 mL-cultured media were collected and radioactivity was determined. " * " indicates this treatment differed significantly ($p<0.0001$) from control (0 nM ZLLL). ZLLL at 100 nM was toxic to the cells. Results represent the mean of two repeated experiments. Triplicates were done in each experiment.

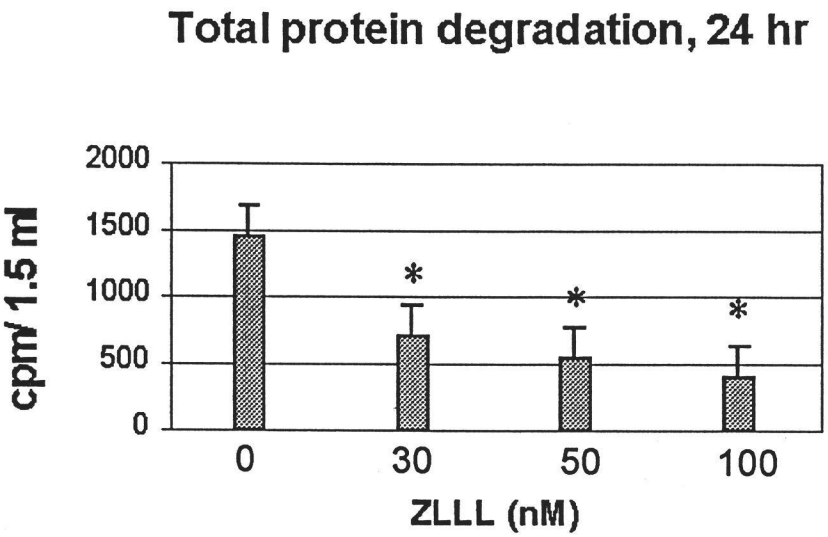


Figure 14. Effect of ZLLL on total protein degradation at 24 hours following serum deprivation in L8/CID myotubes. ^{3}H-tyrosine-prelabeled L8/CID myotubes were incubated with different ZLLL concentrations (0, 30, 50, 100 nM) in DMEM for 24 hours as described in text. After treatments, 1.5 mL-cultured media were collected and radioactivity was determined. " * " indicates this treatment differed significantly ($p<0.0001$) from control (0 nM ZLLL). ZLLL at 100 nM was toxic to the cells. Results represent the mean of two repeated experiments. Triplicates were done in each experiment.

was greater than that effect of 10 mM methylamine. These data demonstrate that a large portion of muscle protein degradation is mediated by lysosomal proteases and the proteasome.

Coordination of the Protease System to Total Protein Degradation

To study the effects of the combination of proteases on protein breakdown, we examined 24-hr total protein degradation with a ^{3}H-tyrosine labeling experiment. Inhibitors tested included 30 nM ZLLL, 10 mM methylamine and 5 mM IPTG. These concentrations were selected based on their inhibitory responses (i.e. not too high to be able to observe the additive effects and were not toxic to the cells). As seen in previous experiments, IPTG caused only a slight reduction in degradation. ZLLL and methylamine either alone or in combination with others significantly inhibited total protein degradation (Figure 15). However, the presence of IPTG with either methylamine or ZLLL did not show greater reduction in protein degradation. The combination of ZLLL and methylamine or three inhibitors together had the highest inhibitory effect (67%). Cell toxicity was observed in the treatments with all inhibitors and IPTG plus methylamine.

Evaluation of the effects of the inhibitors on stability of the myofibrillar and non-myofibrillar protein pools was achieved by measuring ^{3}H-tyrosine remaining in these protein fractions following incubation in the presence of inhibitor or IPTG. The reduction of the myofibrillar and non-myofibrillar protein pool during 24 hr of serum withdrawal was approximately 31% and 38%, respectively. The intracellular pools did

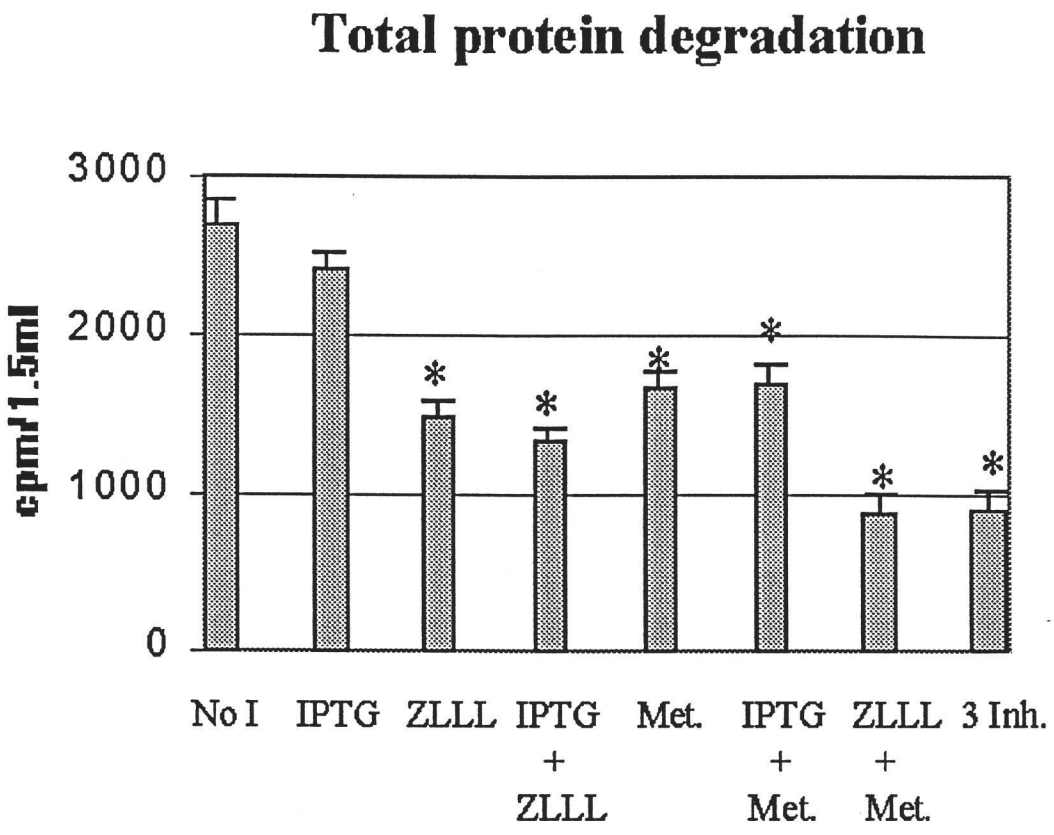


Figure 15. Effects of 24 hr-treatment of various protease inhibitors on total protein degradation following serum withdrawal in L8/CID cell lines. ^{3}H-tyrosine-prelabeled L8/CID myotubes were incubated with different inhibitor concentrations (5 mM IPTG, 30 nM ZLLL and 10 mM methylamine in DMEM for 24 hours as described in text. After treatments, 1.5 mL-cultured media were collected and radioactivity was determined. " * " indicates this treatment differed significantly (*Scheffe* test) from control (no inhibitor). Results represent the mean of three repeated experiments. Triplicates were done in each experiment.

not change significantly following incubation with inhibitors (*Scheffe'* test). We found that methylamine had the greatest effects (+27%) on stabilizing both intracellular pools (Figures 16 and 17). IPTG increased the myofibrillar pool by 4%. ZLLL had slight effects on myofibrillar protein pool size (+10%). IPTG increased the effect of ZLLL on non-myofibrillar pool from 5 to 12% and myofibrillar pool from 10 to 14.5%. These data indicate that the lysosome and proteasome contribute to degradation of total proteins in muscle cells. The net contribution of the proteasome is slightly higher than that of lysosome. Lysosomal function seems essential for the enhanced proteolysis of both protein pools. The ubiquitin-proteasome pathway has a greater effect on the myofibrillar compartment than the non-myofibrillar compartment. The role of calpain in regulation of myofibrillar and non-myofibrillar protein pool size is inconclusive as it had only a slight inhibitory effect on total protein degradation in L8/CID cells.

Stabilization of Muscle Proteins by the Protease Inhibitors

In order to determine whether the various protease systems cooperatively degrade cytoskeletal/myofibrillar proteins, we assessed the degradation of desmin, α-actinin, filamin, tropomyosin and dystrophin in L8/CID cell lines. Differentiated L8/CID cell lines were divided into five groups. The first group was a control group (no treatment). The others were treated with IPTG or methylamine+IPTG, ZLLL+IPTG and ZLLL+methylamine for 48 hr. Serum withdrawal was applied at day 2. At the end of treatment, proteins were extracted and subjected to SDS-PAGE. Western blotting was performed. Lac I concentration was determined as an internal control for equal loading

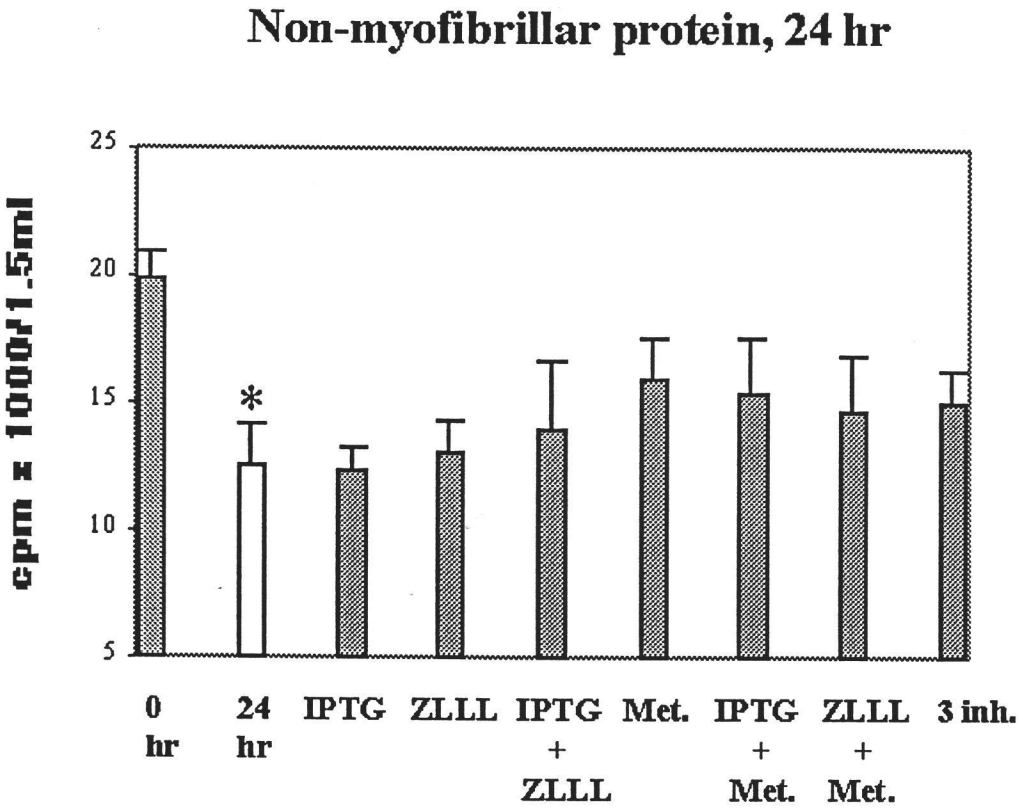


Figure 16. Effects of 24 hr-treatment of various protease inhibitors on stability of non-myofibrillar proteins in L8/CID cell lines. ^{3}H-tyrosine-prelabeled L8/CID myotubes were incubated with different inhibitor concentrations (5 mM IPTG, 30 nM ZLLL and 10 mM methylamine in DMEM for 24 hours as described in text. After treatments, the non-myofibrillar proteins were collected and radioactivity was determined. The non-myofibrillar protein pools of inhibitor-treated cells did not differ significantly from untreated cells at 24 hr following serum withdrawal. " * " indicates statistically significant reduction of non-myofibrillar proteins (no inhibitor) at 24 hr over 0 hr. Results represent the mean of three repeated experiments. Triplicates were done in each experiment.

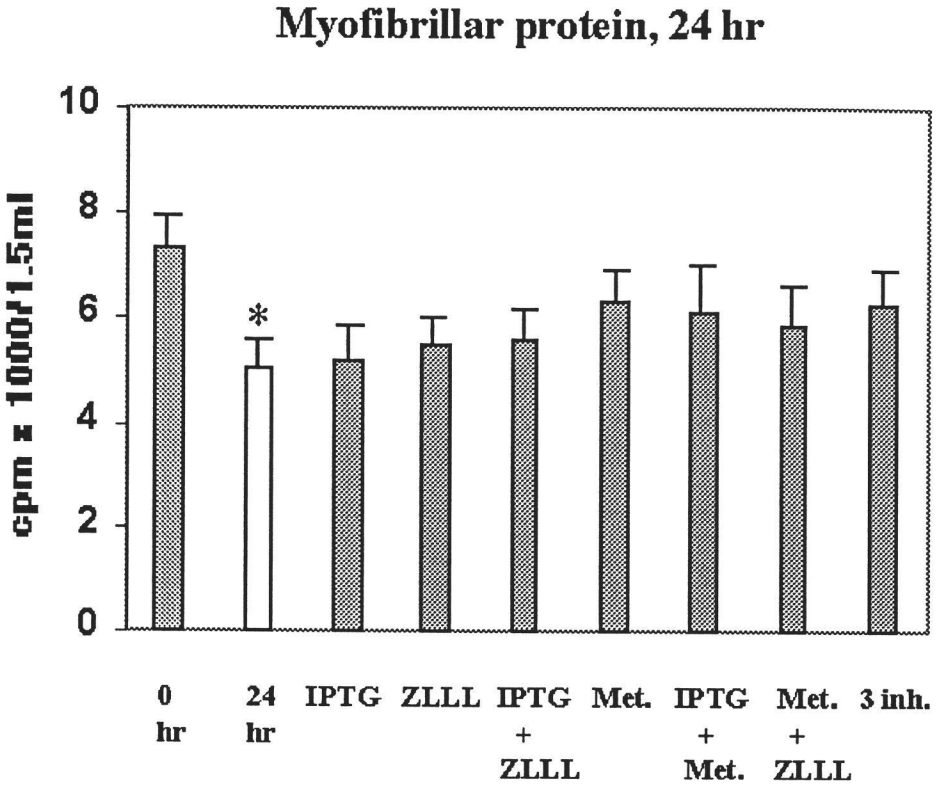


Figure 17. Effects of 24 hr-treatment of various protease inhibitors on stability of myofibrillar proteins in L8/CID cell lines. ^{3}H-tyrosine-prelabeled L8/CID myotubes were incubated with different inhibitor concentrations (5 mM IPTG, 30 nM ZLLL and 10 mM methylamine in DMEM for 24 hours as described in text. After treatments, the myofibrillar proteins were collected and radioactivity was determined. The myofibrillar protein pools of inhibitor-treated cells did not differ significantly from untreated cells at 24 hr following serum withdrawal. " * " indicates statistically significant reduction of myofibrillar proteins (no inhibitor) at 24 hr over 0 hr. Results represent the mean of three repeated experiments. Triplicates were done in each experiment.

(Figure 18). The experiment was repeated twice. The Western blot results showed that inhibition of all proteolytic pathways in muscle cells stabilized m-calpain. The greatest effects were seen in IPTG-treated cells (+60%, Figure 10). After autolytic activation that generates 78-and 28-kDa m-calpain and 76- and 18-kDa polypeptides of μ-calpain, calpain eventually autolyzes to produce 35-, 22-, and 18-kDa fragments for m-calpain and the 55-, 34- and 21-kDa fragments for μ-calpain (Nishimura and Goll, 1991). Calpastatin is a calpain specific inhibitor that binds to and inhibits calpain activity by preventing calpain autolysis (Saido et al., 1994; Goll et al., 1989), thus inhibiting calpain degradation. These results indicate that the lysosomal and proteasome pathways may be responsible for the further degradation of autolyzed calpain in muscle cells.

Western blotting revealed the additive effect on accumulation of desmin (Figure 19: panel A) in IPTG plus ZLLL- or methylamine-treated cells (+181% and +284%, respectively). It seems likely that the effect of methylamine on stabilization of desmin was slightly higher than that of ZLLL. Dystrophin was stabilized by IPTG, ZLLL and methylamine (Figure 19: panel B). Combination of IPTG and either ZLLL or methylamine increased dystrophin concentration (+353% and +376%, respectively). The accumulation of α-actinin and filamin was clearly detected only following treatment with ZLLL plus methylamine (+62% and +11%; Figures 20: panel A and B, respectively). Even though filamin is known to be a substrate of calpain, no difference was observed following the addition of IPTG. Tropomyosin levels were increased in all treatments compared to control (Figure 21). Taken together, results from the inhibitor treatments and Western blot analysis of L8/CID cells indicated the coordination of the

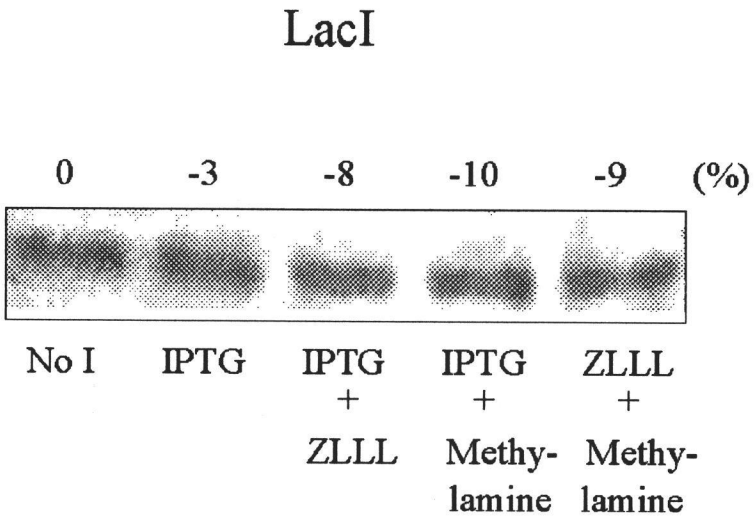
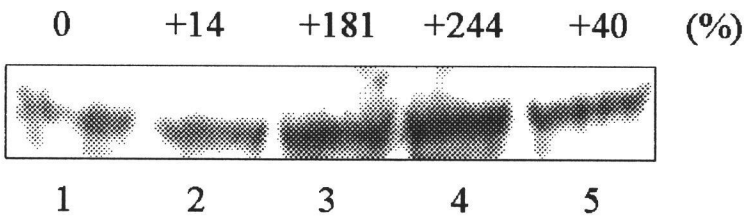


Figure 18. Effect of over-expressed CID, proteasome and lysosomal inhibitors on LacI concentration in L8/CID myotubes. L8/CID cell lines were treated with IPTG, ZLLL and methylamine for 48 hr. Forty μg of each cultured cell lysate protein was assessed by western blot. Polyclonal LacI antibody was used to probe the blot as described in the text. Numbers on top of the blot indicate percentage difference of each band's intensity compared to control (no I).

A: Desmin



B: Dystrophin

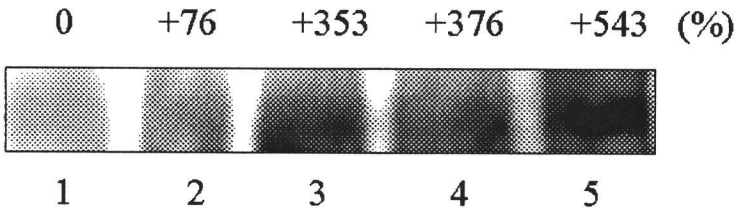


Figure 19. Effect of over-expressed CID, proteasome and lysosomal inhibitors on desmin (panel A) and dystrophin (panel B) concentration in L8/CID myotubes. L8/CID cell lines were treated with IPTG, ZLLL and methylamine for 48 hr. Sixty μg of each cultured cell lysate protein was assessed by western blot. Monoclonal desmin and dystrophin antibodies were used to probe the blots as described in the text. Numbers on top of the blots indicate percentage difference of each band's intensity compared to control (no inhibitor). Numbers belowed the blots indicate the inhibitor treatments. 1: no inhibitor; 2: IPTG; 3: IPTG plus ZLLL; 4: IPTG plus methylamine; 5: ZLLL plus methylamine.

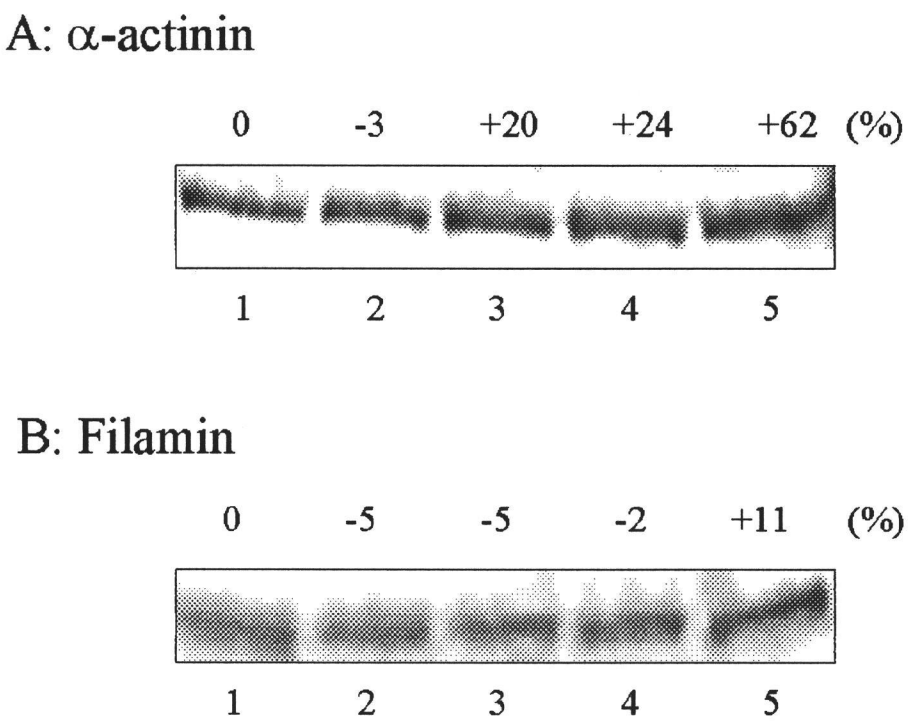


Figure 20. Effect of over-expressed CID, proteasome and lysosomal inhibitors on α-actinin (panel A) and filamin (panel B) concentration in L8/CID myotubes. L8/CID cell lines were treated with IPTG, ZLLL and methylamine for 48 hr. Forty μg of each cultured cell lysate protein was assessed by western blot. Monoclonal α-actinin and filamin antibodies were used to probe the blots as described in the text. Numbers on top of the blots indicate percentage difference of each band's intensity compared to control (no inhibitor). Numbers belowed the blots indicate the inhibitor treatments. 1: no inhibitor; 2: IPTG; 3: IPTG plus ZLLL; 4: IPTG plus methylamine; 5: ZLLL plus methylamine.

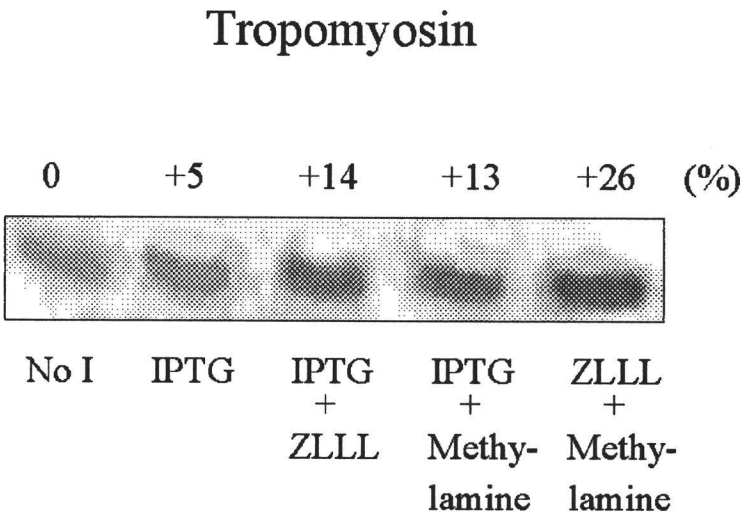


Figure 21. Effect of over-expressed CID, proteasome and lysosomal inhibitors on tropomyosin concentration in L8/CID myotubes. L8/CID cell lines were treated with IPTG, ZLLL and methylamine for 48 hr. Forty μg of each cultured cell lysate protein was assessed by western blot. Monoclonal tropomyosin antibody was used to probe the blot as described in the text. Numbers on top of the blot indicate percentage difference of each band's intensity compared to control (no I).

protease systems in degradation of a variety of muscle proteins. Calpain plays a role in initiating protein degradation of a number of proteins especially desmin and dystrophin. Proteasome and lysosome contribute to degradation of individual proteins into amino acids, particularly desmin, tropomyosin and dystrophin, and are mainly responsible for α-actinin and filamin degradation.

Screening of the Human Skeletal Muscle cDNA Library with m-Calpain cDNA

To screen for proteins able to interact with m-calpain, we used the yeast expression system designed to detect protein-protein interactions as a result of their ability to reconstitute the transactivation function of the GAL4 protein. Thus, dominant negative-80K- and 78Δ4-mCL cDNA (mCL: m-calpain large subunit) were inserted into pAS2-1, fused in-frame to GAL4-BD (see Figure 4). Expression of GAL4-BD/calpain proteins was confirmed by Western blot using antibodies against GAL4-BD (Figure 22). Co-expression of 80K or 78Δ4 with pACT2 (a vector that encodes DNA sequences corresponding to the GAL4-AD) in the yeast Y190 containing a GAL4 binding site that drives expression of *lacZ* did not provide any activation of *lacZ* expression. Fusion of human skeletal muscle cDNA library to the GAL4-AD in pGAD10 led to the appearance of four colonies strongly expressing *lacZ* (Table 2) when transformed into the yeast pre-transformed with 80K or 78Δ4 containing plasmids. Transformation efficiency was $1\text{-}2\text{x}10^3$ colony-forming unit (cfu)/μg DNA and approximately $3\text{-}4\text{x}10^6$ clones were screened. We detected no positive clones from the library screen using other forms of m-calpain (78K, 80Δ4), native m- and μ-calpain

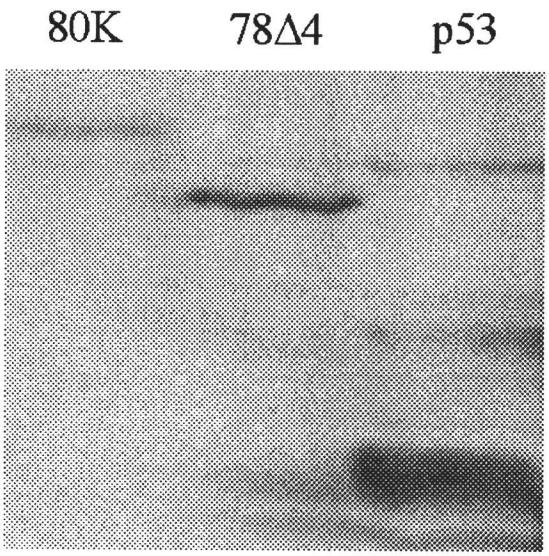


Figure 22. Expression of m-calpain/GAL4-BD hybrid proteins in the yeast strain Y 190. Yeast cell lysates were prepared as described in text from yeast cells expressing mCLDN, 78Δ4 and p53 (positive control). Sixty µl of each lysate was subjected to SDS-PAGE (10%). The membrane was probed with monoclonal antibodies for GAL4-BD.

Table 2. Interaction between calpain and proteins in skeletal muscle cDNA library

In pAS2-1	In pGAD10	β-galactosidase activity (unit)	Growth on SD/-leu,-trp,-his,+3AT
p53	SV 40 large T antigen	179.1±15.85	+
80K	-	0.22±0.12	-
80K	Ca^{2+}-ATPase	25.39±3.35	+
80K	Glycogen phosphorylase	9.34±0.89	+
78Δ4	Nebulin-related protein	22.53±8.59	+
78Δ4	Creatine kinase	5.25±0.17	+
78Δ4	-	0.02±8.86	-

as the baits. Very small background (<5%) could be detected from the μ-calpain screen after 14-day incubation suggesting the toxicity effect of overexpressed μ-calpain.

Verification of Positive Two-Hybrid Interaction

After recovering the plasmids encoding cDNAs which interacted with calpain cDNA, the cDNA plasmids were re-transformed with the bait plasmids into the yeast strain Y187 to reconstitue the GAL4-driven expression of *lacZ*. Unfortunately, we could not detect lacZ expression in any clones. In order to confirm the interaction of fusion proteins by another method, the yeast mating experiments were performed as mentioned previously (Table 1) with and without Ca^{2+} ionophore A 23187 (Sigma) in the media (1 mM final concentration). Again, we could not detect any clones expressing lacZ. We amplified the cDNA inserts by PCR using primers specific to GAL4-AD and pGAD10. The PCR products revealed the nucleotide fragments of approximately 3.9, 2.0, 3.1 and 2.3 kb generated from four different clones (Figure 23). Sequencing results of these cDNAs identified the encoding proteins to be Ca^{2+}-ATPase, novel nebulin-related protein, creatine kinase and glycogen phosphorylase, respectively. The Genbank entry numbers are gb/U96773, gb/U76618, gb/M14780 and gb/M32579-M32598, respectively. From the sequencing profiles, we discovered that all cDNA inserts were not subcloned in-frame to the GAL4-AD. Therefore, the presence of internal stop codon derived from the shift of reading frame causes the early termination of expressed-GAL4-AD/cDNA fusion proteins at nucleotides 79, 19, 67 and 301 downstream of the fusion point (Figure 24). These results indicate that incomplete expression of the fusing

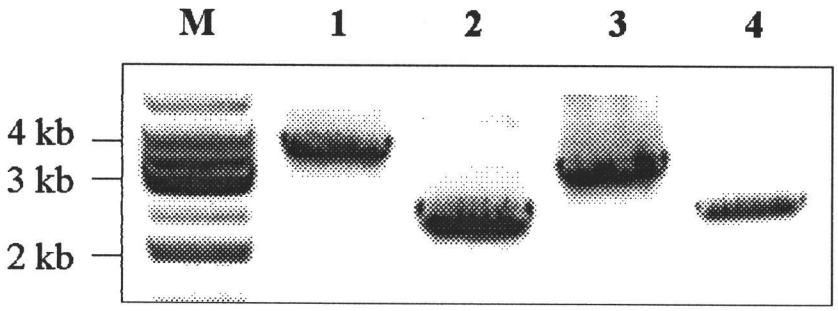


Figure 23. PCR-amplified products of the human skeletal muscle cDNA library plasmids derived from two-hybrid screening with m-calpain. These cDNAs encode Ca^{2+}-ATPase (Lane 1), nebulin-related protein (Lane 2), creatine kinase (Lane 3) and glycogen phosphorylase (Lane 4). Lane M is a 0.5 kb DNA ladder.

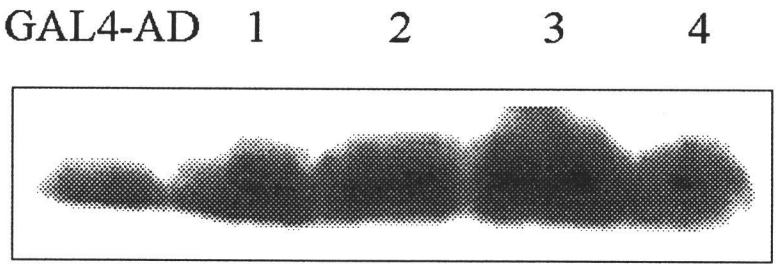


Figure 24. Expression of library/GAL4-AD hybrid proteins in the yeast strain Y 190. Yeast cell lysates were prepared as described in text from yeast cells expressing GAL4-AD only (positive control), Ca^{2+}-ATPase (Lane 1), nebulin-related protein (Lane 2), creatine kinase (Lane 3) and glycogen phosphorylase (Lane 4). Sixty μl of each lysate was subjected to SDS-PAGE (10%). The membrane was probed with monoclonal Ab for GAL4-AD.

cDNAs may be due to confirmation failure or the presence of another factor(s) needed endogenously to mediate the binding of the fusion proteins.

Substrate Cleavage by m-Calpain

To investigate whether the two-hybrid candidates could be cleaved by calpain, the *in vitro* hydrolysis of glycogen phosphorylase and creatine kinase by m-calpain was evaluated. Figure 25 shows the SDS-PAGE pattern of hydrolytic products of creatine kinase after 15 min-incubation with calpain in the presence of Ca^{2+}. We also found that calpain cleaved glycogen phosphorylase into five smaller fragments (Figure 26). Prolonged incubation for 30 min resulted in the increase in the breakdown products. No proteolysis of substrates was observed in the absence of calpain. These results suggested that creatine kinase and glycogen phosphorylase are *in vitro* substrates of m-calpain. We have not yet investigated the two other two-hybrid candidates (Ca^{2+}-ATPase and nebulin-related protein) since we could not find the commercially available sources of these purified proteins.

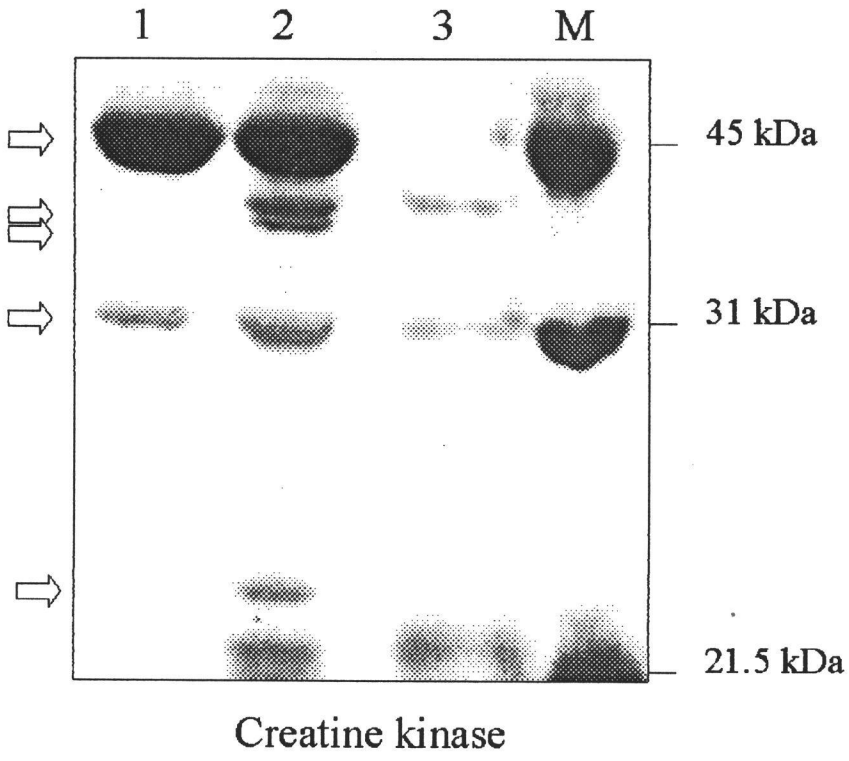


Figure 25. SDS-PAGE (10%) showing the effects of digestion by m-calpain on creatine kinase. Creatine kinase (20 ng) was incubated at 30°C for 15 min in buffer A in the presence of 2 mM $CaCl_2$, without calpain (lane 1) and with calpain (lane 2). Lane 3 shows the reaction mixture with no creatine kinase. The arrows indicate the degraded fragments.

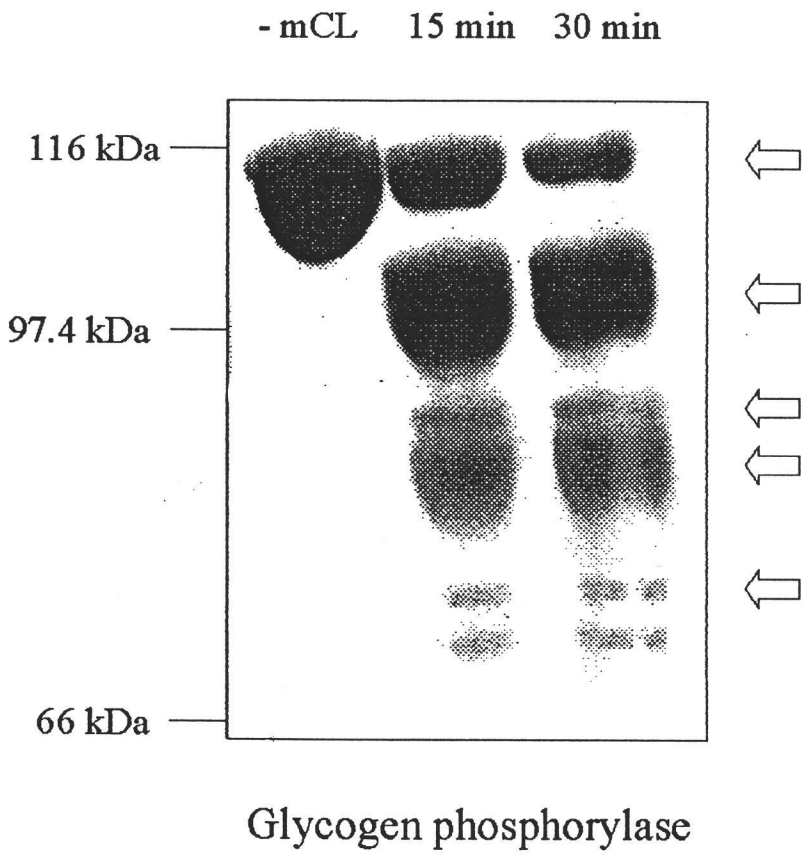


Figure 26. SDS-PAGE (7.5%) showing the effects of digestion by m-calpain on glycogen phosphorylase. Glycogen phosphorylase was incubated at 30°C for the time indicated with 0.5 unit m-calpain in buffer A in the presence of 2 mM $CaCl_2$. The reaction was stopped by addition of excess EDTA. The arrows indicate the degraded fragments.

DISCUSSION

Individual proteins are degraded at widely different rates. For example, while some regulatory proteins have a half-life as short as 15 minute, permitting adaptation to new physiological conditions and changes in cell composition, actin and myosin in skeletal muscle are turning over only once every one to two weeks (Lecker et al., 1999). In conditions such as fasting, metabolic acidosis, muscle denervation, sepsis and cachexia, an increase in proteolysis corresponds to an increase in the protease activities (Furono et al., 1990; Hummel et al., 1998). This response can be blocked with inhibitors of the protease systems (Furono et al., 1990

The present study tested the role of different proteolytic pathways in accelerated-induced muscle proteolysis. There is strong evidence that the induction of the proteolytic pathways contributes to the rapid loss of the contractile proteins observed in several pathological conditions (Tidball 1995; Medina et al., 1991; Wing et al., 1995). Since the protein content of any cell depends on the balance between rates of protein synthesis and degradation, a rise in proteolysis can by itself induce muscle wasting. Our studies suggest that increased Ub-proteasome pathway mainly contributes to the accelerated muscle proteolysis. Lysosomal protease pathway also plays an important role. Clearly, the calpains are not essential to the rise in overall proteolysis (Figure 15). However, it is probably misleading to underestimate the involvement of the calpain system in muscle protein degradation based on the inhibition effect of the CID overexpression. Since the CID expressed in L8/CID cell lines contains only the

polypeptide fragments adopted from a consensus motif of highly conserved amino acid sequences presented in subdomain B of calpastatin inhibitory domain (see Figure 2). Analysis of various mutants and fragments of calpastatin inhibitory domain (Ma et al., 1994) had shown that all three subdomains correlate with potentiation of calpain inhibition. They also found that deletion of subdomains A and B diminishes the calpain-inhibition activity by 50% and completely abolishes calmodulin-like domain binding acitivity of calpastatin. Therefore, the CID expressed from L8/CID cell lines may not process full activity as the whole CID proteins and this may result in the low inhibitory effect observed in our experiments.

The regulatory role of calpastatin on calpain has been studied extensively *in vitro* (for review see Maki et al., 1990). The biological importance of the calpain/calpastatin ratio in cells was demonstrated in myoblast fusion (Barnoy et al., 1996). Our studies suggest that calpastatin regulates calpain level and activity *in vivo.* This finding supports the model for regulation of calpain activity, in which elevated calpastatin levels inhibit an unaltered level of calpain protease (Potter et al., 1998). Calpain subunits are reported to dissociate in the presence of calcium ion (Anagli et al., 1996; Michetti et al., 1997). Calpastatin may keep the two subunits together and stabilize the calpain molecule where the monomeric form is more calcium sensitive but unstable. Autolyzed calpains were reported to disappear very rapidly after activation (Nishimura and Goll, 1991). Our observations that proteasome and lysosome inhibitors stabilize m-calpain indicate that both protease systems are involved in calpain turnover.

In our studies, we found that lysosome can account for large and equal portions of myofibrillar and non-myofibrillar protein breakdown in L8/CID muscle cells. This observation is contrary to a previous report by Lowell et al. (1986) in which evidence was found that lysosomes are not involved in the degradation of myofibrillar protein in rat skeletal muscle. Instead, the Ub-proteasome pathway was mainly responsible for the myofibrillar pool breakdown (Lecker et al., 1999). The factor that may contribute to this contradiction is the cell differentiation rate. The L8/CID myotube half-life is approximately 2-4 days depending on the myoblast confluency at which it was induced to differentiate. Our treatments were completed at day 8 in which some cells (~20%) were shown to detach from the culture plates. Hence, the condition such as cell senescence which is not critical in intact muscle experiments, may be a major interfering factor given that lysosomal pathway is responsible for the turnover of protein in this condition. In addition, our results also indicate that the lysosome contributes non-specifically to overall increased muscle proteolysis which happened during accelerated protein degradation. The findings that calpain increases the effect of myofibrillar protein degradation by the proteasome support the calpain role on limited proteolysis and the role of proteasome on degradation of myofibrillar proteins.

Several lines of evidence suggest that calpain may be the rate-limiting protease in muscle protein degradation. Calpain has limited and specific effects on muscle proteins. Calpains cleave a limited number of proteins and cleave these proteins at a limited number of sites, leaving large polypeptide fragments to be degraded by the other protease systems (Goll et al., 1992a). Calpain initiates the release of thick and thin

filaments from the surface of myofibrils (Goll et al., 1992a) and also rapidly degrades proteins in costameres (Taylor et al., 1995) which results in separation of the sarcolemma from the myofibril (Figure 27). Desmin, filamin, tropomyosin and dystrophin are reported to be degraded by calpain (O'Shea et al., 1979; Davies et al., 1978; Goll et al., 1999; Yoshida et al., 1992). Very little degradation of undenatured α-actinin was observed after prolonged exposure to calpain (Thomson et al., 1993).

Our results that tropomyosin, desmin and dystrophin were stabilized by inhibition of calpain activity indicate that calpain plays a regulatory role in the turnover of these proteins. More importantly, the effects of proteasome and lysosomal inhibitors are additive to the effects of calpain inhibition on desmin and dystrophin stabilization. These findings support the role of calpain in the rate-limiting step in myofibrillar and cytoskeletal protein degradation.

We used the two-hybrid system to search for calpain-binding proteins. The yeast GAL4-based system has both advantages and disadvantages. The GAL4 DNA-binding domain is believed to be more efficiently localized to the nucleus (for review see Bartal et al., 1993). The experiments must be performed in gal4$^-$ yeast strains to avoid background due to activation of the reporter system by endogenous GAL4. The basal expression of *HIS3* can be easily suppressed by 3AT, a chemical inhibitor of the yeast histidine biosynthetic pathway. In the two-hybrid system, the interactions are detected within the natural environment of the cell. However, the use of protein fusion also means that the protein of interest may not fold exactly as the original unfused protein and the site of interaction maybe occluded by one of the transcription factor domains.

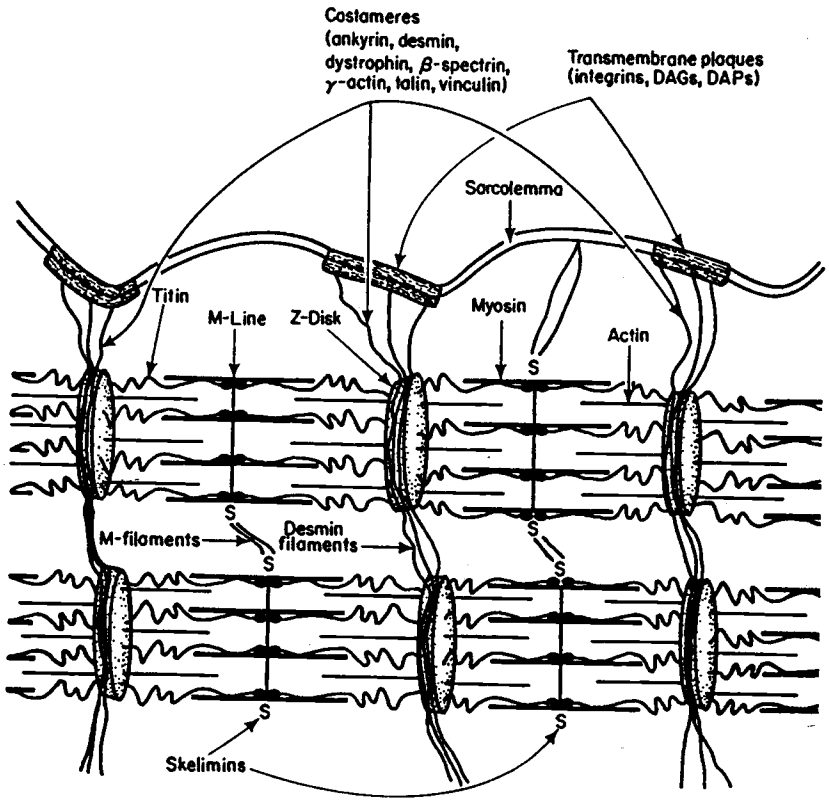


Figure 27. Schematic diagram showing the structure and protein composition of costameres in striated muscle relative to Z-disks and the myofibrillar lattice. The position of titin is shown in this diagram, but nebulin filaments have been omitted to simplify the diagram. The N-terminal end of the large titin molecule is anchored in the Z-disk. Desmin, vinculin and ankylin, three of the protein constituents of costameres, extend into the muscle cell where they encircle myofibrils at the Z-disk and run from myofibril to myofibril to link adjacent Z-disks laterally (Taylor et al., 1995). DAPs = dystrophin-associated proteins; DAG = dystrophin-associated glycans; S = skelemins.

Since false-positive clones caused by non-specific binding is known to be a major problem in yeast cDNA library screening (Luo et al., 1996), it was surprising that we could only detect total of 18 clones that expressed β-galactosidase by repeatedly screening library with different baits. Only 4 out 18 plasmids encoded the known proteins. The remaining were considered to be false positives since they either expressed reporter gene without DNA-BD/calpain fusing proteins or encoding very short sequences with no identity.

Two major points need to be addressed for they may affect the *in vivo* binding of calpain with its interacting partner in the two-hybrid system. First, tissue-purified calpain is phosphorylated. McClelland et al. (1994) reported that m-calpain is phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase only after autolysis indicating that phosphorylation may be necessary for calpain activity. Calpain can be phosphorylated by several protein kinases (Kuo et al., 1993). Hence, interactions dependent on a post-translational modification that does not occur in yeast cells will not be detected. For example, phosphorylation on a tyrosine residue may not occur in yeast (Fields and Sternglanz, 1994). Second, calpastatin binds calpain in a Ca^{2+}-dependent manner. We found that calpastatin did not bind calpain in the yeast two-hybrid system. Calpastatin binds calpain near a catalytic center and mutation of the active site residue of calpain does not interfere calpastatin-binding activity (Crawford et al., 1993). These studies suggest Ca^{2+} concentration is rather low in yeast (Sorimachi et al., 1995) and Ca^{2+}-dependent binding of m-calpain may not occur in the yeast cell.

We considered four proteins (Ca^{2+}-ATPase, nebulin-related protein, creatine kinase (CK) and glycogen phosphorylase) resulting from screening the skeletal muscle cDNA library as potential candidates for *in vivo* substrates of m-calpain. In the cell, CK is bound to intracellular compartments, and is functionally coupled to enzymes and transport systems involved in energy production and utilization (Ventura-Clapier et al., 1998) whereas glycogen phosphorylase is an enzyme involved in glycogenolysis. We have shown that creatine kinase and glycogen phosphorylase are *in vitro* substrates of calpain. This finding suggests that creatine kinase and glycogen phosphorylase interact with m-calpain. m-Calpain may regulate the degradation of creatine kinase and glycogen phosphorylase and possibly regulate their activities.

Nebulin-related protein (N-RAP), a 133-kDa protein, is specifically expressed in skeletal and cardiac muscle. N-RAP is part of a complex of proteins that anchors the terminal actin filaments of the myofibril to the membrane, and functions in transmitting tension from the myofibrils to the extracellular matrix (Figure 28; Luo et al., 1999). There is evidence showing that N-RAP shares extensive similarity with the C-terminal region of human nebulin (Luo et al., 1997). Nebulin is an excellent substrate for the calpain. Calpain rapidly degrades nebulin to a series of smaller fragments which evidently remain bound to actin (Taylor et al., 1995). Based on the structural similarity between nebulin and N-RAP, N-RAP could possibly be cleaved by calpain.

Two studies showed the plasma membrane calcium pump (Ca^{2+}-ATPase) is a preferred μ-calpain substrate within the erythrocyte (Salamino et al., 1994; Molinari et al., 1994). Ca^{2+}-ATPase is stimulated by interaction with calmodulin at the site close to

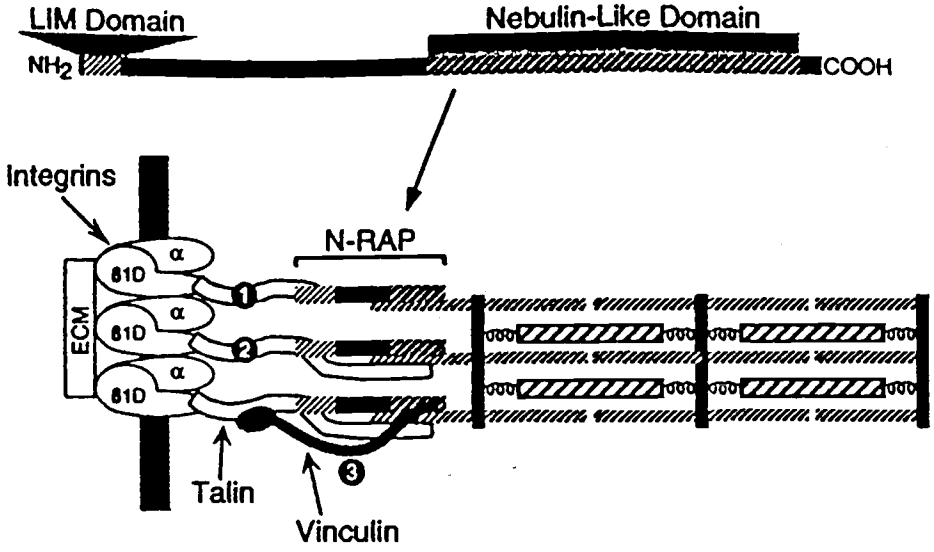


Figure 28. Possible schematic diagram for linking the terminal actin filaments of myofibrils to the cell membrane. The domain organization of N-RAP is shown at the top, with distinct hatch patterns marking the positions of the nebulin-related region and the LIM domain. These domains are shown binding to the terminal actin filaments and to talin, respectively; talin in turn binds to the β1D integrin subunit, completing the linkage between the myofibril and the plasma membrane (numeral 1). Interactions between talin and actin filament (numeral 2) and between vinculin and talin, actin and N-RAP (numeral 3) are sequentially added. The integrins transmit tension produced by cytoplasmic structures to the extracellular matrix (ECM, Luo et al., 1999).

its C-terminus. In the absence of calmodulin, calpain cleaves the purified Ca^{2+}-ATPase at the calmodulin-binding domain resulting in an active 124 kDa-polypeptide (James et al., 1989). However, in the *in vivo* environment, the end result of calpain action is the complete inactivation of the pump. These observations suggest that m-calpain, a closely related isoform of μ-calpain may also interact with Ca^{2+}-ATPase and may regulate Ca^{2+}-ATPase activity and degradation *in vivo*.

Since μ-calpain and m-calpain have very similar if not identical subsite specificities, they may degrade the same protein substrates in *in vitro* assays (Goll et al., 1999). For example, calpain prefers Val or Leu in the P2 position and less stringent at the P1 position (Sasaki et al., 1984). The presence of the secondary recognition sequence(s) adjacent to the cleavage sites dictates substrate's susceptibility to calpain (Wang et al., 1989; Molinali et al., 1995). Consequently, it is reasonable to assume that μ- and m-calpain are capable of cleaving the same substrates in living cells.

Additional studies are needed to learn whether m-calpain associates with and/or cleaves these proteins *in vivo*. The incomplete expression of candidate cDNAs retrieved from the two-hybrid clones can be overcome by correcting the reading-frames or replacing the screened-cDNA with the full-length cDNA sequences of the human Ca^{2+}-ATPase, nebulin-related protein, creatine kinase and glycogen phosphorylase genes (if available). The actual protein-protein interaction should be verified by performing the yeast two-hybrid system. Alternatively, the interactions of the protein hybrids can be confirmed by immunoprecipitation, ELISA using antibodies against the yeast GAL4, calpain or proteins of interest and by affinity column chromatography. If an interaction

is detected, deletions can be made in the DNA encoding either calpain or its partner to identify a minimal domain for interaction. In addition, point mutations can be assayed to identify specific amino acid residues critical for the interaction. The findings may provide the role of calpain in regulation of protein degradation and function of calpain in the cell.

CONCLUSION

Serum withdrawal increased myofibrillar and non-myofibrillar protein degradation in L8/CID muscle cells by 31 and 38%, respectively. The inhibition of cellular protease systems reduced total protein degradation by approximately 67%. The protease systems including the lysosomal, Ub-proteasome and calpain systems which coordinately regulated intracellular proteolysis. Ub-proteasome and lysosomal pathways account for most protein degradation. Lysosomes contributed non-selectively to cellular protein turnover. The Ub-proteasome system appears mainly responsible for the myofibrillar pool degradation. Calpain plays definite roles in protein degradation in muscle cells. Calpain is important for initiating degradation of myofibrillar, cytoskeletal proteins and a number of individual proteins especially desmin and dystrophin. Inhibition of calpain stabilized tropomyosin, desmin and dystrophin. Inhibition of proteasome and lysosome also stabilized tropomyosin, desmin and dystrophin as well and stabilized other proteins such as α-actinin and filamin indicating the involvement of the Ub-proteasome and lysosomal pathways in the turnover of these proteins.

By screening skeletal muscle cDNA library using the yeast two-hybrid system, four partial cDNAs which encoded creatine kinase, glycogen phosphorylase, Ca^{2+}-ATPase and nebulin-related protein were identified as potential candidates of m-calpain-binding proteins. Purified creatine kinase and glycogen phosphorylase were cleaved by m-calpain in an *in vitro* assay. We have not yet evaluated whether Ca^{2+}-ATPase and nebulin-like protein also serve as substrates for calpain.

BIBLIOGRAPHY

Allen, R. E. (1988) Muscle cell growth and development. *In* Designing foods animal product options in the market place. p 142-162. National Research Concil, National Acad. Press, Washington D. C.

Anagli, J., Vilei, E. M., Molinali, M., Calderara, S. and Carafoli, E. (1996) Purification of active calpain by affinity chromatography on an immobilized peptide inhibitor. *Eur. J. Biochem.* 241, 948-954.

Balcerzak, D., Patrick, C., Poussard, S., Cucuron, A., Brustis, J. J. and Ducastaing, A. (1998) Calpastatin-modulation of m-calpain activity is required for myoblast fusion. *Eur. J. Cell Biol.* 75, 247-253.

Baracos, V. E., DeVivo, C., Hoyle, D. H. and Goldberg, A. L. (1995) Activation of the ATP-ubiquitin proteasome pathway in skeletal muscle of cachectic rats bearing a hepatoma. *Am. J. Physiol.* 268, E996-E1006.

Barnoy, S. T., Glasner, T. and Kosower, N. S. (1996) The role of calpastatin (the specific calpain inhibitor) in myoblast differentiation and fusion. *Biochem. Biophys. Res. Comm.* 220, 933-938.

Barnoy, S., Glaser, T. and Kosower, N. S. (1998) The calpain-calpastatin system and protein degradation in fusing myoblasts. *Biochimica et Biophysica Acta* 1402, 52-60.

Bartal, P. L., Chien, C.-T., Sternglanz, R. and Fields, S. (1993) Using the two-hybrid system to detect protein-protein interactions. *In* Cellular Interactions in Development: A Practical Approach (Hartley, D. A., ed.) p 153-179. Oxford University Press, Oxford.

Bartal, P. L., Roecklein, J. A., SenGupta, D. and Fields, S. (1996) A protein linkagemap of *Escherichia coli* bacteriophage T7. *Nature Genet.* 12, 72-77.

Bates, P. C. and Millward, D. L. (1983) Myofibrillar protein turnover. *Biochem. J.* 214, 587-592.

Belcastro, A. N., Shewchuk, L. D. and Raj, D. (1998) Exercise-induced muscle injury: a calpain hypothesis. *Mol. Cell. Biochem.* 179, 135-145.

Bond, J. S. and Barrett, A. J. (1980) Degradation of fructose-1,6-bisphosphate aldolase by cathepsin B. *Biochem. J.* 189, 17-25.

Chardin, P., Camonis, J.H., Gale, N. W., Aelst, V. L., Schlessinger, J., Wigler, M. H. and Bar-Sagi, D. (1993) Human Sos1: a guanine nucleotide exchage factor for Ras that binds to GRS2. *Science* 260, 1338-1343.

Cong, J.-Y., Goll, D. E., Peterson, A. M. and Kapprell, H-P. (1989) The role of autolysis in activity of the Ca^{2+}- dependent proteinases (μ-calpain and m-calpain). *J. Biol. Chem.* 264, 10096-10103.

Cong, M., Thompson, V. F., Goll, D. E. and Antin, P. (1998) The bovine calpastatin gene promoter and a new N-terminal regoin of the protein are targets for cAMP dependent protein kinase activity. *J. Biol. Chem.* 273, 660-666.

Crawford, C., Brown, N. R. and Willis, A. C. (1993) Studies of the active site of m-calpain and the interaction with calpastatin. *Biochem. J.* 296, 135-142.

Davies, P. J. A., Wallach, D., Willingham, M. C., Pastan, I., Yamaguchi, M. and Robson, R. M. (1978) Filamin-actin interaction. Dissociation of binding from gelatin by Ca^{2+}-activated proteolysis. *J. Biol. Chem.* 253, 4036-4042.

Dayton, W. R. (1982) Comparison of low- and high-calcium-requiring forms of the calcium-activated protease with their autocatalytic breakdown products. *Biochem. Biophys. Acta* 709, 166-172.

Ding, X., Price, S. R., Bailey, J. L. and Mitch, W. E. (1997) Cellular mechanisms controlling protein degradation in catabolic states. *Miner Electrolyte Metab.* 23, 194-197.

Drees, B. L. (1999) Progress and variations in two-hybrid and three-hybrid technologies. *Curr. Opin. Chem. Biol.* 3, 64-70.

Durfee, T., Becherer, K., Chen, P. L., Yeh, S. H., Yang, Y., Kilbburn, A. E., Lee, W. H. and Elledge, S. J. (1993) The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes Devel.* 7, 555-569.

Ebisui, C., Tsujinaka, T., Morimoto, T. Kan, K., Iijima, S. Yano, M., Kominami, E., Tanaka, K. and Monden, M. (1995) Interleukin-6 induces proteolysis by activating intracellular proteases (cathepsins B and L, proteasome) in C2C12 myotubes. *Clin. Sci. (Colch)* 89, 431-439.

Emori, Y., Kawasaki, H., Imajoh, S., Imahori, K. and Suzuki, K. (1987) Endogenous inhibitor for calpain-dependent cysteine protease contains four internal repeats that could be responsible for its multiple reactive sites. *Proc. Natl. Acad. Sci.* 84, 3590-3594.

Estojak, J., Brent, R. and Golemis, E. A. (1995) Correlation of two-hybrid affinity data with in vitro measurements. *Mol. Cell Biol.* 15, 5820-5829.

Etlinger, J. D. and Goldberg, A. L. (1977) A soluble ATP-dependent proteolytic system responsible for the degradation of abnormal proteins in reticulocytes. *Proc. Natl. Acad. Sci. USA* 74, 54-58.

Fang, C. H., Tiao, G., James, H., Ogle, C., Fisher, J. E. and Hasselgren, P. O. (1995) Burn injury stimulates multiple proteolytic pathways in skeletal muscle, including the ubiquitin-energy-dependent pathway. *J. Am. Coll. Surg.* 180, 161-170.

Farah, C. S. and Reinach, F. C. (1995) The troponin complex and regulation of muscle contraction. *The FASEB Journal* 9, 755-767.

Fernandez, C. and Sainz, R. D. (1997) Pathways of protein degradation in L6 myotubes. *Proc. Soc. Exp. Biol. Med.* 214, 242-247.

Fields, S. and Song, O. (1989) A novel genetic system to detect protein-protein interactions. *Nature* 340, 245-246.

Fields, S. and Sternglanz, R. (1994) The two-hybrid system: an assay for protein-protein interactions. *Trends Genet.* 10, 286-292.

Figueiredo-Pereira, M. E., Banik, N. and Wilk, S. (1994) A new inhibitor of the chymotrypsin-like activity of the multi-catalytic proteinase complex (20S proteasome) induces accumulation of ubiquitin-protein conjugates in a neuronal cell. *J. Neuro. Chem.* 62, 1989-1994.

Fleischer, S. and Inui, M. (1989) Biochemistry and biophysics of excitation-contraction coupling. *Annu. Rev. Biophys. Biophys. Chem.* 18, 333-364.

Forsberg, N. E., Ilian, M. A., Al-Bar, A., Checke, P. R. and Wehr, N. B. (1989) Effects of cimaterol on rabbit growth and myofibrillar protein degradation and on calcium-dependent proteinase and calpastatin activities in skeletal muscle. *J. Anim. Sci.* 67, 3313-3321.

Furono, K., Goodman, M. N. and Goldberg, A. L. (1990) Role of different proteolytic systems in the degradation of muscle proteins during denervation atrophy. *J. Biol. Chem.* 265, 8550-8557.

Geesink, G. H., Nonneman, D. and Koohmaraie, M. (1998) An improved purification protocol for heart and skeletal muscle calpastatin reveals two isoforms resulting from alternative splicing. *Arch. Biochem. Biophys.* 356, 19-24.

Gietz, D., St. Jean, A., Woods, R. A. and Shiestl, R. H. (1992) Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res.* 20, 1425.

Goldberg, A. L. and St. John, A. C. (1976) Intracellular protein degradation in mammalian and bacterial cells: part 2. *Ann. Rev. Biochem.* 45, 747-803.

Goldstein, G., Steed, M., Hammerling, U., Boyse, E. A., Schlesinger, D. H. and Niall, H. D. (1975) Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells. *Proc. Natl. Acad. Sci. USA* 72, 11-15.

Goll, D. E., Kleese, W. C. and Szpacenko, A. (1989) Skeletal muscle proteases and protein turnover. *In* Animal growth regulation (Campion, D. R., Hausman, G. J., Martin, R. J., eds.) p 141-182.

Goll, D. E., Thompson, V. F., Taylor, R. G. and Zalewska, T. (1992b) Is calpain activity regulated by membranes and autolysis or by calcium and calpastatin. *BioEssays* 14, 549-556.

Goll, D. E., Thompson, V. F., Taylor, R. G., Ouali, A. and Chou, R.-G. (1999) The calpain system in muscle tissue. *In* Calpain, pharmacology and toxicology of calcium-dependent protease (Wang, K. K. and Yuen, P.-W., eds.) p 127-160. Taylor & Francis, Philadelphia, PA.

Goll, D. E., Thomson, V. F., Taylor, R. G. and Chrisstiansen, J. A. (1992a) Role of the calpain system in muscle growth. *Biochimie* 74, 225-237.

Goll, D. E., Dayton, W. R., Singh, I. and Robson, R. M. (1991) Studies of the α-actinin/actin interaction in the Z-disk by using calpain. *J. Biol. Chem.* 266, 8501-8510.

Gordon, S. R. and DeMoss, J. (1999) Exposure to lysosomotropic amines and protease inhibitors retard corneal endothelial cell migration along the natural basement membrane during wound repair. *Exp. Cell Res.* 246, 233-242.

Gregory, J. W. and Bihovsky, R. (1998) Calpain inhibitors as potential treatment for stroke and other neurodegenerative diseases: recent trends and developments. *Exp. Opin. Ther. Patents* 8, 1707-1727.

Guan, K., Jenkins, C., Li, Y., Nochols, M. A., Wu, X., O'Keefe, C. L., Matera, A. G. and Xiong, Y. (1994) Growth suppression by p18, a $p16^{INK4/MTS1}$ and $p14^{INK4B/MTS2}$-related CDK6 inhibitor, correlates with wild-type pRb function. *Genes Devel.* 8, 2939-2952.

Guroff, G. and Guroff, G. (1964) A neutral, calcium-activated proteinase from the soluble fraction of rat brain. *J. Biol. Chem.* 239, 149.

Guthrie, C. and Fink, G. R. (1991) Guide to yeast genetics and molecular biology. In *Methods in enzymology* (Academic Press, San Diego) 194, 1-932.

Hanada, K., Tamai, M., Yamagishi, M. Ohmura, S. Sawada, J. and Tanaka, I. (1978) Isolation and characterization of E-64, a new thiol protease inhibitor. *Agric. Biol. Chem.* 42, 523-528.

Harkins, A. B., Kurebayashi, N. and Baylor, S. M. (1993) Resting myoplastic free calcium in frog skeletal muscle fibers estimated with fluo-3. *Biophys. J.* 65, 865-881.

Heilig, J. S., Elbing, K. L. and Brent, R. (1994) Large-scale preparation of plasmid DNA. *In* Current protocols in molecular biology: Vol 1. (Ausubel, et al., editors). John Wiley & Sons, Inc., Brooklyn, NY.

Higgins, J. A., Lasslett, Y., Bardsley, R. G. and Buttery, P. J. (1988) The relation between dietary restriction or clenbuterol (a selective β2 agonist) treatment on muscle growth and calpain proteinase (EC3.4.22.17) and calpastatin activities in lambs. *Brit. J. Nutr.* 60, 645-652.

Hill, J., Donald, K. A. and Griffiths, D. E. (1991) DMSO-enhanced whole cell yeast transformation. *Nucleic Acids Res.* 19, 5791.

Hong, D.-H. and Forsberg, N. E. (1994) Effects of serum and insulin-like growth factor I on protein degradation and protease gene expression in rat L8 myotubes. *J. Anim. Sci.* 72, 2279-2288.

Hong, S-C., Goto, Y., Lanzino, G., Saleau, S., Kassel, N. F. and Lee, K. S. (1994) Neuroprotection with a calpain inhibitor in a model of focal cerebral eschemia. *Stroke* 25, 663-669.

Huang, J. and Forsberg, N. E. (1998) Role of calpain in skeletal-muscle protein degradation. *Proc. Natl. Acad. Sci. USA* 95, 12100-12105.

Hummel, R. P. 3rd., James, J. H., Warner, B. W., Hasselgren, P. O. and Fischer, O. H. (1998) Evident that cathepsin B contributes to skeletal muscle protein breakdown during sepsis. *Arch. Surg.* 123, 221-224.

Imajoh, S., Kawasaki, H., Emori, Y. and Suzuki, K. (1987) Calcium-activated neutral protease inhibitor from rabbit erythrocytes lacks the N-terminal region of the liver inhibitor but retains three inhibitory units. *Biochem. Biophys. Res. Comm.* 146, 630-632.

Inomata, M., Hayashi, M., Nakamura, M., Saito, Y. and Kawashima, S. (1989) Properties of erythrocyte membrane binding and autolytic activation of calpain activated neutral protease. *J. Biol. Chem.* 264, 18838-18843.

Ishiura, S., Sugita, H., Nonaka, I. and Imahori, K. (1980) Calcium-activated neutral protease, its localization in the myofibril, especially at the Z-band. *J. Biochem.* 87, 343-346.

Ito, H., Fukada, Y., Murata, K. and Kimura, A (1983) Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* 153, 163-168.

James, P., Vorherr, T., Krebs, J., Morelli, A., Castello, G., McCormick, D. J., Penniston, J. T., De Flora, A. and Carafoli, E. (1989) Modulation of erytrohcyte Ca^{2+}-ATPase by selective calpain cleavage of the calmodulin-binding domain. *J. Biol. Chem.* 264, 8289-8296.

Jensen, T. J., Loo, M. A., Pind, S., Williams, D. B., Goldberg, A. L. and Riordan, J. R. (1995) Multiple proteolytic systems, including the proteasome, contribute to CFTR processing. *Cell* 83, 129-135.

Kapprell, H.-P. and Goll, D. E. (1989) Effect of Ca^{2+}on binding of the calpain to calpastatin. *J. Biol. Chem.* 264, 17888-17896.

Kato, G. J., Lee, W. M. F., Chen, L. and Dang, C. V. (1992) Max: functional domains and interaction with c-Myc. *Genes Devel.* 6, 81-92.

Kawasaki, H. and Kawashima, S. (1996) Regulation of calpain-calpastatin system by membranes (review). *Mol. Membr. Biol.* 13, 217-224.

Kinbara, K., Sorimachi, H., Ishiura, S. and Suzuki, K. (1998) Skeletal muscle-specific calpain, p94: structure and physiological function. *Biochem. Pharmacol.* 56, 415-420.

Kisselev,A. F., Akopian, T. N. and Goldberg, A. L. (1998) Range of sizes of peptide products degenerated during degradation of different proteins by archaeal proteasomes. *J. Biol. Chem.* 273, 1982-1989.

Koomaraie, M. (1992) Ovine skeletal muscle multicatalytic proteinase complex (proteasome) purification, characterization and comparison of its effects on myofibril with μ-calpain. *J. Anim. Sci.* 70, 3697-3708.

Koomaraie, M. and Shackelford, S. D. (1991) Effect of calcium chloride infusion on the tenderness of lambs fed a β-adrenergic agonist. *J. Anim. Sci.* 69, 2463-2471.

Kuboki, M., Ishii, H. and Kazama, M. (1990) Characterization of calpain I-binding proteins in human erythrocyte plasma membrane. *J. Biochem.* 107, 776-780.

Kumamoto, T., Kleese, W. C., Cong, J. Y., Goll, D. E., Pierce, P. R. and Allen, R. E. (1992) Localization of the Ca^{2+}-dependent proteinases and their inhibitor in normal, fasted, and denervated rat skeletal muscle. *Anat. Rec.* 232, 60-77.

Kuo, W. N., Ganesan, U., Walbey, D. L., Davis, D. L., Allen, K. and McCall, L. K. (1993) Modulation of the activity of calpain II by phosphorylation—changes in the proteolysis of cyclic AMP-dependent protein kinase (peak II, DEAE). *Appl. Theor. Electrophor.* 3, 317-320.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Lecker, S. H., Solomon, V., Mitch, W. E. and Goldberg, A. L. (1999) Muscle protein breakdown and the critical role of the ubiquitin-proteasome pathway in normal and disease states. *J. Nutr.* 129, 227S-237S.

Lee, W. J., Hatanaka, M. and Maki, M. (1992) Multiple forms of rat calpastatin cDNA in the coding region of functionally unknown amino terminal domain. *Biochem. Biophys. Acta* 1129, 251-253.

Lee, W. J., Ma, H., Takano, E., Yang, H. Q., Hatanaka, M. and Maki, M. (1992b) Molecular diversity in amino-terminal domains of human calpastatin by exon skipping. *J. Biol. Chem.* 267, 8437-8442.

Li, B. and Fields, S. (1993) Identification of mutations in p53 that affect its binding to SV40 T antigen by using the yeast two-hybrid system. *FASEB J.* 7, 957-963.

Lin, G. D., Chattopadhyay, D., Maki, M., Wanh, K. K., Carson, M., Jin, L., Yuen, P. W., Takano, E., Hatanaka, M., DeLucas, L. J. and Narayana, S. V. (1997) Crystal structure of calcium bound domain VI of calpain at 1.9 A resolution and its role in enzyme assembly, regulation, and inhibitor binding. *Nat. Strcut. Biol.* 4, 539-547.

Lowell, B. B., Ruderman, N. B. and Goodman, M. N. (1986) Evidence that lysosomes are not involved in the degradation of myofibrillar proteins in rat skeletal muscle. *Biochem. J.* 234, 237-240.

Lowry, O. H., Rosebrough, A. L., Farr, A. L. and Randall, R. L. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193, 265-268.

Luo, G., Herrera, A. H. and Horowits, R. (1999) Molecular interactions of N-RAP, a nebulin-related protein of striated muscle myotendon junctions and intercalated disks. *Biochemistry* 38, 6135-6143.

Luo, G., Zhang, J. Q., Nguyen, T. P., Herrera, A. H., Paterson, B., Horowits, R. (1997) Complete cDNA sequence and tissue localization of N-RAP, a novel nebulin-related protein of striated muscle. *Cell Motil. Cytoskeleton* 38, 75-90.

Luo, Y., Vijaychander, S., Stile, J. and Zhu, L. (1996) Cloning and analysis of DNA-binding proteins by yeast one-hybrid and one-two-hybrid systems. *BioTechniques* 20, 564-568.

Ma, H., Hata, I., Shih, M., Fukiage, C., Nakamura, Y., Azuma, M. and Shearer, T. R. (1999) Lp82 is the dominant form of calpain in young mouse lens. *Exp. Eye Res.* 68, 447-456.

Ma, H., Yang, H. Q., Takano, E., Hatanaka, M. and Maki, M. (1994) Amino-terminal conserved region in proteinase inhibitor domain of calpastatin potentiates its calpain inhibitory activity by interacting with calmodulin-like domain of the proteinase. *J. Biol. Chem.* 269, 24430-24436.

Maki, M., Hatanaka, M., Takano, E. and Murachi, T. (1990) Structure-function relationship of calpastatins. *In* Intracellular calcium-dependent proteolysis (Mellgren, R. L. and Murachi, T., eds.), p 37-54. CRC Press, Boca Raton, FL.

Maki, M., Takano, E., Mori, H., Sato, A., Murachi, T. and Hatanaka, M. (1987) All four internally repetitive domains of pig calpastatin process inhibitory activity against calpains I and II. *FEBS Lett.* 223, 174-180.

Mansoor, O., Beaufrere, B., Boirie, Y., Ralliere, C., Taillandier, D., Aurousseau, E., Schoeffler, P., Arnal, M. and Attaix, D. (1996) Increased mRNA levels for components of the lysosomal, Ca^{2+}-activated and ATP-ubiquitin-dependent proteolytic pathways in skeletal muscle from head trauma patients. *Proc. Natl. Acad. Sci. USA* 93, 2714-2718.

McClelland, P., Adam, L. P. and Hathaway, D. R. (1994) Identification of a latent Ca2+/calmodulin dependent protein kinase II phosphorylation site in vascular calpain II. *J. Biochem. (Tokyo).* 115, 41-46.

Medina, R., Wing, S. S., Haas, A. and Goldberg, A. L. (1991) Activation of the Ub-ATP-dependent proteolytic system in skeletal muscle during fasting and denervation atrophy. *Biomed. Biochim. Acta* 50, 347-356.

Melloni, E., Michetti, M. Salamino, F. Sparatore, B. and Pontremoli, S. (1998) Mechanism of action of a new component of the Ca^{2+}-dependent proteolytic system in rat brain: the calpain activator. *Biochem. Biophys. Res. Commun.* 249, 583-588.

Meyer, W. L., Fisher, E. H. and Krebs, E. G. (1964) Activation of skeletal muscle phosphorylase b kinase by calcium. *Biochemistry* 3: 1033.

Michetti, M., Salamino, F., Minafra, R., Melloni, E. and Pontremoli, S. (1997) Calcium-binding properties of human erythrocyte calpain. *Biochem. J.* 325, 721-726.

Michetti, M., Voitti, P. L., Meloni, E. and Pontremoli, S. (1991) Mechanism of action of the calpain activator protein in rat skeletal muscle. *Eur. J. Biochem.* 202, 1177-1180.

Millward, D. C. (1980) Protein degradation in muscle and liver. *Comp. Biochem.* 19B, 153-232.

Minor, L. K., Mahlberg, F. H., Jerome, W. G., Lewis, J. C., Rothblat, G. H. and Glick, J. M. (1991) Lysosomal hydrolysis of lipids in a cell culture model of smooth muscle foam cells. *Exp. Mol. Pathol.* 54, 159-171.

Mitch, W. E., Medina, R., Grieber, S., May, R. C., England, B. K., Price, S. R., Bailey, J. L. and Goldberg, A. L. (1994) Metabolic acidosis stimulates muscle protein degradation by activating the ubiquitin and proteasomes. *J. Clin. Invest.* 93, 2127-2133.

Molinari, M., Anagli, J. and Carafoli, E. (1994) Ca^{2+}-activated neutral protease is active in the erythrocyte membrane in its nonautolyzed 80-kDa form. *J. Biol. Chem.* 269, 27992-27995.

Molinari, M., Maki, M. and Carafoli, E. (1995) Purification of μ-calpain by a novel affinity chromatography approach. New insights into the mechanism of the interaction of the protease with targets. *J. Biol. Chem.* 270, 14576-14581.

Myers, B. M., Tietz, P. S. Tarara, J. E. and LaRusso, N. F. (1995) Dynamic measurements of the acute and chronic effects of lysosomotropic agents on hepatocyte lysosomal pH using flow cytometry. *Hepatology* 22, 1519-1526.

Nawaz, Z., Lonard, D. M., Dennis, A. P., Smith, C. L. and O'Malley, B. W. (1999) Proteasome-dependent degradation of the human estrogen receptor. *Proc. Natl. Acad. Sci. USA* 96, 1858-1862.

Nishimura, T. and Goll, D. E. (1991) Binding of calpain fragments to calpastatin. *J. Biol. Chem.* 266, 11842-11850.

O'Shea, J. M., Robson, R. M., Huiatt, T. W., Hartzer, M. K. and Stromer, M. H. (1979) Purified desmin from adult mammalian skeletal muscle: A peptide mapping comparison with desmin from adult mammalian and avian smooth muscle. *Biochem. Biophy. Res. Comm.* 89, 972-980.

Ono, Y., Sorimachi, H. and Suzuki, K. (1998) Structure and physiology of calpain, an enigmatic protease. *Biochem. Biophy. Res. Commun.* 245, 289-294.

Otto, H.-H. and Schirmeister, T (1997) Cysteine proteases and their inhibitors. *Chem. Rev.* 97, 133-171.

Parr, T., Bardsley, R. G., Gilmour, R. S. and Buttery, P. J. (1992) Changes in calpain and calpastatin mRNA induced by β-agonist stimulation of bovine skeletal muscle. *Eur. J. Biochem.* 208, 333-339.

Phizicky, E. M. and Fields, S. (1995) Protein-protein interactions: methods for detection and analysis. *Microbiol. Rev.* 59, 94-123.

Pontremoli, S., Salamino, F., Sparatore, B., Michetti, M., Sacco, O. and Melloni, E. (1985) Following association to the membrane, human erythrocyte procalpain is converted and released as fully active calpain. *Biochimica et Biophysica Acta* 831, 335-339.

Potter, D. A., Tirnauer, J. S., Janssen, R., Croall, D. E., Hughes, C. N., Fiacco, K. A., Mier, J. W., Maki, M. and Herman, I. M. (1998) Calpain regulates actin remodeling during cell spreading. *J. Cell Biol.* 141, 647-662.

Pringle, T. D., Calkins, C. R., Koomaraie, M. and Jones, S. J. (1993) Effects over time of feeding a β-adrenergic agonist to wether lambs on animal performance, muscle growth, endogenous muscle proteinase activities and meat tenderness. *J. Anim. Sci.* 71, 636-644.

Quinn, P. and Judah, J. (1978) Calcium-dependent golgi-vesicle fusion and cathepsin B in the conversion of proalbumin into albumin in rat liver. *Biochem. J.* 172, 301-309.

Rock, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L. Hwang, D. and Goldberg, A. L. (1994) Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell* 78, 761-771.

Saido, T. C., Sorimachi, H. and Suzuki, K. (1994) Calpain: new perspectives in molecular diversity and physiological-pathological involvement. *FASEB J.* 8, 814-822.

Saido, T. C., Suzuki, H., Yamazaki, H., Tanoue, K. and Suzuki, K. (1993) In situ capture of mu-calpain activation in platelets. *J. Biol. Chem.* 268, 7422-7426.

Salamino, F., Sparatore, B., Melloni, E., Michetti, M., Viotti, P. L., Pontremoli, S. and Carafoli, E. (1994) The plasma membrane calcium pump is the preferred calpain substrate within the erythrocyte. *Cell Calcium* 15, 28-35.

Sasaki, T., Kikuchi, T., Yumoto, N. and Murachi, T. (1984) Comparative specificity and kinetic studies on porcine calpain I and calpain II with naturally occuring peptides and synthetic fluorogenic substrates. *J. Biol. Chem.* 259, 12489-12494.

Schiestl, R. H. and Gietz, R. D. (1989) High efficiency transformation of intact cells using single stranded nucleic acids as a carrier. *Curr. Genet.* 16, 339-346.

Scornik, O. A., Howell,S. K. and Botbol, V. (1997) Protein depletion and replenishment in mice: Different roles of muscle and liver. *Am. J. Physiol.* 273, 1158-1167.

Shinozaki, K., Maruyama, K., Kume, H., Tomita, T., Saido, T. C., Iwatsubo, T. and Obata, K. (1998) The presenilin 2 loop domain interacts with the mu-calpain C-terminal region. *Int. J. Mol. Med.* 1, 797-799.

Shoshan-Barmatz, V., Weil, S., Meyer, H., Varsanyi, M. and Heilmeyer, L. (1994) Endogenous, Ca^{2+}-dependent cysteine-protease cleaves specifically the ryanodine receptor/Ca^{2+} release channel in skeletal muscle. *J. Membr. Biol.* 142, 281-288.

Shuster, C. B. and Herman, I. R. (1995) Indirect association of ezrin with F-actin: isoform specificity and calcium sensitivity. *J. Cell Biol.* 128, 837-848.

Solomon, V. and Goldberg, A. L. (1996) Importance of the ATP-ubiquitin-proteasome pathway in the degradation of soluble and myofibrillar proteins in rabbit muscle extracts. *J. Biol. Chem.* 271, 26690-26697.

Sorimachi, H., Imajoh-Ohmi, S., Emori, Y., Kawasaki, H., Ohno, S., Minami, Y. and Suzuki, K. (1989) Molecular cloning of a novel mammalian calcium-dependent protease distinct from both m- and μ-types. Specific expression of the mRNA in skeletal muscle. *J. Biol. Chem.* 264, 20106-20111.

Sorimachi, H., Ishiura, S, and Suzuki, K. (1993) A novel tissue-specific calpain species expressed predominantly in the stomach comprises two alternative splicing products with and without a Ca^{2+}-binding domain. *J. Biol. Chem.* 268, 19476-19482.

Sorimachi, H., Ishiura, S. and Suzuki, K. (1997) Structure and physiological function of calpains. *Biochem. J.* 328, 721-732.

Sorimachi, H., Kinbara, K., Kimura, S., Takahashi, M., Ishiura, S., Sasagawa, N., Soromashi, N., Shimada, H., Tagawa, K., Maruyama, K. and Suzuki, K. (1995) Muscle-specific calpain, p94, responsible for limb girdle muscular dystrophy type 2A, associates with connectin through IS2, a p94 specific sequence. *J. Biol. Chem.* 270, 31158-31162.

Sorimachi, H., Ohmi, S., Emori, Y., Kawasaki, H., Saido, T. C., Ohno, S., Minami, Y. and Suzuki, K. (1990) A novel member of the calcium-dependent cysteine protease family. *Biol. Chem. Hoppe-Seyler* 371, 171-176.

Suzuki, K. (1990) The structure of the calpains and the calpain gene. *In* Intracellular calcium-dependent proteolysis (Mellgren, R. L. and Murachi, T., eds.), p 25-35. CRC Press, Inc., Boca Raton, Florida.

Suzuki, K., Sorimachi, H., Yoshizawa, T., Kinbara, K. and Ishiura, S (1995) Calpain: novel family members, activation, and physiologic function. *Biol. Chem. Hoppe-Seyler* 376: 523-529.

Taillandier, D., Aurousseau, E., Meynial-Denis, D., Bechet, D., Ferrara, M., Cottin, P., Ducastaing, A., Bigard, X., Guezennec, C.-Y., Schmid, H.-P. and Attaix, D. (1996) Coordinate activation of lysosomal, Ca^{2+}-activated and ATP-ubiquitin-dependent proteinases in the unweighted rat soleus muscle. *Biochem. J.* 316, 65-72.

Takano, E., Ma, H., Yang, H. Q., Maki, M. and Hatanaka, M. (1995) Preference of calcium-dependent interactions between calmodulin-like domains of calpain and calpastatin subdomains. *FEBS lett.* 362, 93-97.

Taylor, R. G., Geesink, G. H., Thompson, V. F., Koohmaraie, M. and Goll, D. E. (1995) Is Z-disk degradation responsible for postmortem tenderization?. *J. Anim. Sci.* 73, 1351-1367.

Temparis, S., Asensi, M., Taillandier, D., Aurousseau, E., Larbaud, D., Obled, A., Bechet, D., Ferrara, M., Estrela, J. M. and Attaix, D. (1994) Increased ATP-ubiquitin-dependent proteolysis in skeletal muscle of tumor-bearing rats. *Cancer Res.* 54, 5568-5573.

Thompson, V. F., Taylor, R. G., Goll, D. E., Huff, E. J. and Robson, R. M. (1993) Effect of μ- and m- calpain on bovine skeletal muscle myofibrils and IZI brushes. *Molecular Biology of the cell* 4, 386a.

Tiao, G., Fagan, J. M., Samuels, N., Howard, J., Hudson, K., Lieberman, M., Fischer, J. E. and Hasselgren, P.-O. (1994) Sepsis stimulates nonlysosomal, energy-dependent proteolysis and increases ubiquitin mRNA levels in rat skeletal muscle. *J. Clin. Invest.* 94, 2255-2264.

Tidball, J. D. (1995) Inflammatory cell response to acute muscle injury. *Med. Sci. Sports Exerc.* 27, 1022-1032.

Tischler, M. E., Rosenberg, S., Satarug, S., Henriksen, E. J., Kirby, C. R., Tome, M. and Chase, P. (1990) Different mechanisms of increased proteolysis in atrophy induced by denervation or unweighting of rat soleus muscle. *Metabolism* 39, 756-63.

Tsubuki, S., Saito, Y., Tomioka, M., Ito, H. and Kawashima, S. (1996) Differential inhibition of calpain and proteasome activities by peptidyl aldehydes of di-leucine and tri- leucine. *J. Biochem, (Tokyo)* 199, 572-576.

Turner, P. R., Fong, P., Denetclaw, W. F. and Steinhardt, R. A. (1991) Increased calcium influx in dystrophic muscle. *J. Cell Biol.* 115, 1701-1712.

Ventura-Clapier, R., Kuznetsov, A., Veksler, V., Boehm, E. and Anflous, K. (1998) Functional coupling of creatine kinases in muscles: Species and tissue specificity. *Mol. Cell. Biochem.* 184, 231-247.

Vilei, E. M., Calderara, S., Anagli, J., Berardi, S., Hitomi, K., Maki, M. and Carafoli, E. (1997) Functional properties of recombinant calpain I and of mutants lacking domains III and IV of the catalytic subunit. *J. Biol. Chem.* 272, 25802-25808.

Villa, P. G., Henzel, W. J., Sensenbrenner, M. and Henderson, C. E. (1998) Calpain inhibitors, but not caspase inhibitors, prevent actin proteolysis and DNA fragmentation during apoptosis. *J. Cell Sci.* 111: 713-722.

Voet, D. and Voet, J. G. (1995) Biochemistry: 2nd edition, p 1242-1243. John Wiley & Sons, Somerest, NJ.

Voisin, L., Breuille, D., Combaret, L., Pouyet, C., Taillandier, D., Aurousseau, E., Obled, C. and Attaix, D. (1996) Muscle wasting in a rat model of long-lasting sepsis results from the activation of the lysosomal, Ca^{2+}-activated and ubiquitin-proteasome proteolytic pathways. *J. Clin. Invest.* 97, 1610-1617.

Vojtek, A., Hollenberg, S. and Cooper, J. (1993) Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell* 74, 205-214.

Wang, K. K. and Yuen, P-W. (1994) Calpain inhibition: an overview of its therapeutic potential. *Trends Pharmacol. Sci.* 15, 412-419.

Wang, K. K. W. and Yuen, P.-W. (1999) *In* Calpain: Pharmacology and Toxicology of Calcium-Dependent Protease (Wang, et al., eds.), 448 pp. Taylor & Francis, Philadelphia, PA.

Wang, K. K., Nath, R., Posner, A., Raser, K. J., Buroker-Kilgore, M., Hajimohammadreza, I., Probert, A.W., Jr., Warcoux, F. W., Ye, Q., Takano, E., Hatanaka, M., Maki, M., Caner, H., Collins, J. L., Fergus, A., Lee, K. S., Lunney, E. A., Hays, S. J. and Yuen, P. (1996a) An alphamercaptoacrylic acid derivative is a selective nonpeptide cell-permeable calpain inhibitor and is neuroprotective. *Proc. Natl. Acad. Sci.* 93, 6687-6692.

Wang, K. K., Posner, A. and Hajimohammadreza, I. (1996b) Total protein extraction from cultured cells for use in electrophoresis and western blotting. *BioTechniques* 20, 662-668.

Wang, K. K. W., Villalobo, A. and Roufogalis, B. D. (1989) Calmodulin binding protein as calpain substrates. *Biochem. J.* 262, 693-706.

Wang, S. Y. and Beermann, D. H. (1988) Reduced calcium-dependent proteinase activity in cimaterol-induced muscle hypertrophy in lambs. *J. Anim. Sci.* 66, 2545-2550.

Weinstein, R. B., Slentz, M. J., Webster, K. Takeuchi, J. A. and Tischler, M. E. (1997) Lysosomal proteolysis in distally or proximally denervated rat soleus muscle. *Am. J. Physiol.* 273, R1562-R1565.

Weinstein, R. B., Slentz, M. J., Webster, K. Takeuchi, J. A. and Tischler, M. E. (1997) Lysosomal proteolysis in distally or proximally denervated rat soleus muscle. *Am. J. Physiol.* 273, R1562-R1565.

Whipple, G. and Koohmaraie, M. (1991) Degradation of myofibrillar proteins by extractable lysosomal enzymes and m-calpain, and the effects of zinc chloride. *J. Anim. Sci.* 69, 4449-4460.

Wing, S. S. and Goldberg, A. L. (1993) Glucocorticoids activate the ATP-ubiquitin-dependent proteolytic system in skeletal muscle during fasting. *Am. J. Physiol.* 264, E668- E676.

Wing, S. S., Haas, A. L. and Goldberg, A. L. (1995) Increase in ubiquitin-protein conjugates concomitant with the increase in proteolysis in rat skeletal muscle during starvation and atrophy denervation. *Biochem. J.* 307, 639-645.

Yang, Y. T. and McElligott, M. A. (1989) Multiple actions of β-adrenergic agonists on skeletal muscle and adipose tissue. *Biochem. J.* 261, 1-10.

Yoshida, M., Suzuki, A., Shimizu, T. and Ozawa, E. (1992) Proteinase-sensitive sites on isolated rabbit dystrophin. *J. Biochem.* 112, 433-439.

Yoshizawa, T., Sorimachi, H., Tomioka, S., Ishiura, S. and Suzuki, K. (1995) A catalytic subunit of calpain processes full proteolytic activity. *FEBS Letters* 358, 101-103.

Young, K. H. (1998) Yeast two-hybrid: so many interactions, (in) so little time. *Biol. Reprod.* 58, 302-311.

Young, V. R., Steffee, W. P., Pencharz, P. B. Winterer, J. C. and Scrimshaw, N. S. (1975) Total human body protein synthesis in relation to protein requirements at various ages. *Nature* 253, 192-193.