AN ABSTRACT OF THE THESIS OF

Ying-Yi Xiao for the degree of Doctor of Philosophy in Animal Science and Molecular & Cellular Biology presented on March 15, 2001. Title: Regulation of Skeletal Muscle Protein Degradation by u-Calpain and Development of a Skeletal Muscle-Specific Inducible Expression System

Abstract approved: Redacted for Privacy

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The first goal of this study was to understand the role of u-calpain in skeletal muscle protein degradation in cultured muscle cells. Several strategies were developed to down-regulate endogenous u-calpain activity and m-calpain activity in rat myotubes. These included over-expression of antisense u-calpain (AnsL), dominant negative u-calpain (DN-u-CL), antisense 30K subunit (AnsS) and fused antisense u-calpain/30K (AnsLS, i.e., 80K/30K). The ability to regulate calpain activity was confirmed by fodrin degradation (an index of calpain activity). Our data supported the contention that u-calpain contributes significantly to total protein degradation in myotubes. Specifically, over-expressing DN-u-calpain reduced total protein degradation by 7.9% ($P<0.01$) at 24 hr time point and by 10.6% ($P<0.01$) at a 48 hr time point. Similarly, over-expression of antisense u-CL and the 30K subunit reduced total protein degradation significantly at the 24 hr time point ($P<0.05$). However, over-expression of the fused antisense (80K/30K) did not affect ($P>0.05$) the total protein degradation. In addition to this we determined that desmin was a calpain substrate and that calpain could not degrade tropomyosin.

The second goal of this study was to evaluate the relationships among u- and m-calpain and the 30KD subunit. The rationale for this study was that our earlier work indicated coordinated regulation of the calpain subunits. Our data demonstrated for the first time that the transcription and translation of u-calpain and 30K, and m-calpain and 30K are

coordinately regulated, respectively. However, the expression of u-calpain did not affect the expression of m-calpain

The third goal of this study was to develop a skeletal muscle-specific inducible expression system that may be used in transgenic animal research. A skeletal muscle a-actin promoter was used to replace the cytomegalovirus immediate-early promoter (pCMV) in the ecdysone inducible mammalian expression system. LacZ was used as a reporter gene. A beta-galactosidase staining assay and high-sensitivity B-gal activity assay indicated that the skeletal muscle-specific expression system functioned in myotubes. After 48 hr of administration of ponasterone A (inducer), the treated cells had 15-fold higher B-gal activity than the control cells.

Regulation of Skeletal Muscle Protein Degradation by u-Calpain and Development of a Skeletal Muscle-Specific Inducible Expression System

By

Ying-Yi Xiao

A THESIS

submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Doctor of Philosophy

Presented March 15, 2001

Commencement June 2002

Doctor of Philosophy thesis of Ying-Yi Xiao presented on March 15, 2001

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ACKNOWLEDGMENTS

I would like to thank Drs. Moore, Hu, Leid, Ream, Menino, Whanger and Schmisseur for serving on my program committee. All of them play their critical roles for supporting me to complete this project.

I also would like to thank the generous supports from different labs for letting me use their equipment: Dr. Leid's lab, Dr. Hu's lab, Dr. Ream's lab, Dr. Menino's lab, Dr. Whanger's lab and Dr. Stormshak's lab.

Many thanks for the support from Department of Animal Sciences and Molecular & Cellular Biology Program.

I'd like to specially thank Dr. Meyer for his considerate, strong support and precious friendship.

Appreciation is extended to my labmates, Paiyen, Mike, Jing, Meichuan, Yoji, Yung-Hae, Juntipa and Fang for their unlimited support and their wonderful friendship.

Dear Dr. Forsberg, I would like to thank you for the strong support, the broad guidance, the encouragement, the wonderful project and your friendship. I cherish all of these in my lifetime.

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DEDICATION

This Doctoral Dissertation is dedicated to my parents, Shienchi Xiao and Furong Chen, my wife, Wentao Yang, my daughter Yangyu Xiao (Karen), and my son Beracah Xiao. I couldn't finish this study without all of your strong support. I am grateful for your love.

Regulation of Skeletal Muscle Protein Degradation By μ-Calpain and Development of a Skeletal Muscle-Specific Inducible Expression System

Chapter I Introduction

Meat, especially skeletal muscle, is the major product from domestic animals. There are various ways to improve meat production efficiency from meat animals. Appropriate amounts of growth hormone (GH) are helpful for enhancing animal growth (1,2). Growth hormone is considered essential for postnatal somatic growth in domestic animals. GH functions via stimulating synthesis and secretion of insulin-like growth factors (IGFs). And, the stimulatory myogenic effect of IGFs is mediated by their abilities to transcriptionally induce myogenin mRNA (3). Despite the success of exogenous hormones in stimulating growth, a genetic approach might be more efficient than injecting GH into animals. Myostatin (also called growth and differentiation factor-8, GDF-8) is a member of the transforming growth factor-β family (4). Myostatin-null mice show a dramatic and widespread increase in skeletal muscle mass due to the increase in number of muscle fibers (hyperplasia) and thickness of fibers (hypertrophy). Belgian Blue and Piedmontese breeds of cattle, which are characterized by an increase in muscle mass (double muscling), have mutations in their myostatin coding sequence (5, 6, 7).

Enhancing muscle growth could also be achieved by reducing skeletal muscle protein degradation *in vivo*. Muscle proteins turn over with half-lives ranging from 2 to 10 days (8) or 5 to 10% per day. And, it has been estimated that 15 to 25% of the food ingested by animals is used to replace the metabolically turned-over muscle protein (9). According to the physiological condition of animals, this replacement portion could be even higher (10, 11). Genetically reducing protein degradation/turnover in skeletal muscle is a means by which we may enhance animal growth and therefore to improve the feed efficiency.

Inhibition of calpain II (calcium-dependent papain-like protease) has been shown to reduce protein degradation in rat skeletal muscle *in vitro* by 30% (12). A more intriguing observation is that over-expression of calpastatin inhibitory domain, which inhibits both u- and m-calpain (13, 14), could reduce the total protein degradation by 63% (12). The later observation can be equally interpreted as "inhibition of u-calpain could reduce the total skeletal muscle protein degradation by about 30%". This interpretation, however, needs to be strictly tested.

In order to challenge the above "interpretation", appropriate experimental systems should be utilized. In this study, we were interested in the function of u-calpain on total skeletal muscle protein degradation. A second (long term) goal of this study was to develop an expression system which could be applied in vivo and which would allow us to understand u-calpain function in vivo. For this purpose, the ideal expression system should be inducible and tissue-specific. The commercial expression vectors usually are either non-inducible or inducible but not tissue-specific. In order to constrain the genetic change to skeletal muscle; we also planned to modify the expression system to be skeletal muscle-specific.

Calpain and Skeletal Muscle Protein Degradation

Calpain superfamily: The calpains (EC3.4.22.17) are a super-family of calcium-dependent cytosolic non-lysosomal cysteine proteinases (15). Because of their diversities in expression pattern, protein composition and dependency on calcium concentration for their half-maximal activity, the members of this super-family show extremely broad physiological and pathological function in various organisms from fungi to human (16-20). According to their structure, the calpains are classified as conventional calpains and "atypical" calpains. Recently, due to the efforts from the international calpain-research community, the physiological and pathological functions of calpains have become much clear.

The conventional calpains include u-calpain (micro-) or calpain I, m-calpain (milli-) or calpain II, and regulatory subunit 30K or calpain 4 (21). m-calpain and u-calpain form heterodimers with the 30k small subunit. Both m- and u-calpain large subunits could be divided into four domains: a propeptide domain (I), a cysteine protease domain (II), a regulatory domain (III), and a calmodulin-like calcium-binding domain (IV). The 30k subunit can be divided into an N-terminal glycine-rich domain (V) and a calmodulin-like calcium-binding domain (VI). Some other conventional calpains might not form heterodimers with 30K and are expressed tissue-specifically. These include skeletal muscle-specific calpain p94 (22), a lens-specific splicing variant of p94, Lp82 and Lp85 (23, 24), stomach-specific calpains nCL-2 and -2' (25), digestive organ-specific calpain nCL-4 (26), placenta-specific species CAPN6 (27), sol and Dm-calpain (28, 29) from *D. melanogaster,* p71, p72 and p92 from *C. elegans* (30, 31).

Activating mechanism of calpains: Calpains exist in the cytosol as inactive proenzymes. In vitro activation of u- and m-calpains requires ~10-50 uM and ~300-500 uM, respectively, which is considerably higher than physiological Ca^{2+} concentrations (generally <1 uM) (32). Therefore in vivo, calpains must be sensitized to Ca^{2+} and be activated before they achieve their physiological roles. Three factors have been established as potential regulators of Ca^{2+} sensitivity of calpains: autolysis, phospholipids and the 30K subunit (33).

The 30K subunit functions as a chaperone to stabilize the large subunits (u- and m-) in vivo and in vitro (34, 35). Without binding with 30K, the intact or autolyzed large subunits are quickly degraded. Purification of large subunits as monomers from biological resources has never been successful. Based on the X-ray structure of rat (36) and human (37) m-calpain in the absence of Ca^{2+}, plus the information from the crystal structure of Ca^{2+}-binding 30K homodimer (38, 39), the mystery of calpain activation has been clarified. Without Ca^{2+} binding, m-calpain binds with 30K subunit. However, the active site in Domain I and the active sites in the Domain II are prevented from forming an active site cleft. The N-terminal of Domain I is an α-helix anchor that tethers Domain I to Domain VI in the regulatory subuint and plays a key role in the activation process.

Ca^{2+} binding causes conformational changes in Domain IV and Domain VI, which may allow the transducer (in Domain III) to release constraints on other Domains, and exert more flexibility to Domain-II. This might cause the movement of Domain II to Domain III. Spontaneously, the release of the anchor yields a more flexible Domain I. The relief of the restraints from Domain-I and -II would allow formation of the active site cleft at the interface of Domain -I and -II via the rotations to each other. Following the formation of the active site, intermolecular autolysis of the N-terminal anchor of Domain I activates calpain by permitting activity at lower Ca^{2+}-concentration. C_2-like Domain III may be responsible for promoting binding of active calpain to membranes in response to Ca^{2+}, thereby relieving inhibition from calpastatin and promoting digestion of calpain substrates (36).

This activation mechanism might help to resolve seemingly contradictory observations. One is that the 30K subunit dissociates from the large subuint in the presence of Ca^{2+} for the activation of large subunits. The other is that calpain remains as a heterodimer during catalysis (for stabilizing the conformation of large subunits and avoiding from the quick degradation) (40, 41,42).

Despite progress in understanding calpain activation mechanisms, conflicts still exist regarding the activation of m- and u-calpain. Some reports found out that proteolysis can occur without large subunit autolysis of m-calpain (35, 43, 44). In order to compromise with the low physiological Ca^{2+} concentration, some reports suggested that activated u-calpain can activate m-calpain (45, 46). Others report that u-calpain cannot activate m-calpain (47). There are also specific activators for sensitizing m- and u-calpains to Ca^{2+}. UK114 modulates sensitivity of u-calpain to Ca^{2+}(48) and acyl-CoA-binding protein modulates m-calpain sensitivity to Ca^{2+} (49).

Physiological and pathological function of calpains: The exact physiological functions of calpains remain to be clarified. At this time we know that their physiological and pathological functions are just as diverse as their diverse expression patterns. Many pathological functions have been identified and are often linked with the deregulation at

their protein level. Calpain 3, or p94, has drawn broad attention since the discovery that defects in the p94 gene causing loss of p94 activity lead to the development of limb-girdle muscular dystrophy type-2A (50). p94 knock-out mice provided a similar conclusion (51). Two research groups recently independently identified deficiency of gastric-specific calpain nCL4 is related to carcinogenesis (52, 53). Len-specific calpains Lp82 and Lp85 participate in cataractogenesis (23, 24). The *Caenorhabditis elegans* calpain homologue Tra3 determines female sexual development (70). m- and u-calpain and 30K subunit are ubiquitously expressed. Hence, their function should be much broader than their tissue specific counterparts.

The most significant report concerning the function of the 30K calpain subunit is the lethal phenomenon that occurs at the mid-gestation in 30K knock-out (K/O) mouse embryos (19,54). Both u- and m-calpain activities are abolished in 30K K/O embryo. This deletion, however, didn't impact the survival and proliferation of cultured embryonic stem cells or embryonic fibroblasts, even in early stages of organogenesis. The 30K K/O embryos, which died at mid-gestation, showed defects in the cardiovascular system, hemorrhaging, and accumulation of erythroid progenitors (55). The serious consequences to deletion of the 30 KD subunit are based on the spontaneous deletion of both activities of u- and m-calpain. Hence, subsequent double deletion of u- and m-calpain activities accounted for the embryo development defects in 30K K/O, rather than from a direct effect arising from the deletion of 30K. Aside from its chaperon-like function in stabilizing u- and m-calpains, at this time there are no direct physiological or pathological functions assigned to the 30K subunit.

Based on results of the 30KD knock-out experiments, we can anticipate that calpains have widespread and essential functions. The substrate specificity for u- and m-calpains may or may not be similar. Because the activity of calpains is dependent on the concentration of Ca^{2+}, any physiological condition which disturbs Ca^{2+} homeostasis (increase or decrease) has potential to regulate calpains. Similarly, over-expression of calpains will change their activity as well. In spinal cord injury and traumatic brain injury, influx of Ca^{2+} increases calpain activity. Excessive active calpain digests

cytoskeletal proteins (e.g., spectrin) and neurofilament proteins (NFP) (56, 57, 58). Oxidative stress in central neuronal cell has a similar effect (59). Calpain inhibitors, e.g., E-64-D and AK295, can prevent neural damage by inhibiting calpain activity in spinal cord injury and traumatic brain injury. There is also an opposite report stating that behavioral efficacy of post-traumatic calpain inhibition is not accompanied by reduced spectrin proteolysis, cortical lesion, or apoptosis (55). Calpain might participate in the pathogenesis of Alzheimer's disease in two ways. In one way, presenilin (PS)-1 and -2 can decrease m-calpain activity, but not u-calpain. Active u-calpain and m-calpain can degrade presenilin PS-1 and -2 and this will reduce the negative effect of PS-1 and -2 on m-calpain activity (60). In the other proposed mechanism, active u- and m-calpains proteolyze cyclin-dependent kinase 5 (cdk5) activator p35 to p25 (61, 62, 63). p25 causes prolonged activation and mislocalization of cdk5. Consequently, the p25/cdk5 kinase hyperphosphorylates tau, disrupts the cytoskeleton and promotes the death (apoptosis) of primary neurons. u-calpain may even play a role in learning and memory by its interrelation with protein kinase Cγ (64).

The reported functions of u- and m-calpain in apoptosis are not consistent. Calpain inhibitors are usually utilized to elucidate calpain's physiological and pathological function. Calpain can degrade p53. This means that calpain can prevent wild-type p53 cells, but not mutated or null p53 cells, from entering apoptosis (65). E-64-d can prevent both calpain up-regulation and apoptosis in the lesion and penumbra following spinal cord injury in rat (66). However, calpain inhibitor II (CPI-2) induces caspase-dependent apoptosis in acute lymphoblastic leukemia and in non-Hodgkin's lymphoma as well as some solid tumor cells (67). Calpain inhibitor I (CPI-1) can activate p53-dependent apoptosis (65). The discrepancy among these reports might be explained by the differences between experiment systems, (i.e., spinal cord injury and tumor cell lines). More possibly, this discrepancy may be caused by the side effects of the different calpain inhibitors. u-calpain might play a more important role than m-calpain in apoptosis (68,69).

The physiological functions of u- and m-calpains on the metabolic turnover of myofibrillar proteins is more interesting for animal scientists. Past research indicates that calpains may be rate-limiting proteases. Myofibrils in skeletal muscle are too large to be engulfed by lysosomes and the central cavity of the proteasome is too small for intact myofibrils to enter (76). Hence, myofibrils must first be detached from the sarcomere, then be degraded by the proteasome (77). Calpains have very unique and limited specificity for digesting myofibrillar/cytoskeletal proteins, e.g., desmin (71), troponin I and T (72), and spectrin (19). Immunolocalization studies detected calpain and calpastatin on the Z-disk and the I-band of the sarcomere (73, 74). Moreover, incubation with purified calpain results in the loss of Z-disk and the periodicity along the length of the thin filament. (75). The myosin-containing A-bands and the thick filaments remained intact. Therefore, calpains disassemble the myofibrils in skeletal muscle. And, the rate-limiting relationship between calpains and skeletal muscle growth is further indicated by studies on the skeletal muscle growth-enhancement caused by β-adrenergic agonists: it was reported that skeletal muscle calpastatin (endogenous inhibitor of calpains) activity is increased significantly by β-agonist (78, 79,80), but the activities of u- and m-calpain were not significantly affected (78, 80). Even more direct evidence comes from our lab (19). Huang et al. reported that over-expression of calpastatin inhibitory domain (CID) in rat L8 myotube reduced 63% of total skeletal muscle protein degradation. Similarly, over- expression of active site-mutated m-calpain (dominant negative (DN) calpain II) reduced total skeletal muscle protein degradation by 30%. In that study, CID was expected to inhibit both u- and m-calpains, whereas DN-m-calpain was expected to interfere only with m-calpain avtivity. Hence, the difference between effects of CID and DN expression may approximate the contribution of u-calpain to turnover.

Based on the previous rationale, we designed several experiments to test the hypothesis that down-regulation of u-calpain will decrease total skeletal muscle protein degradation in rat L8 cells. To reduce calpain activity we used several strategies. These included:

a) Over-expression of dominant negative u-calpain. This construct was expected to express a "dead" protease which would compete for substrate with endogenous u-calpain and thereby reduce u-calpain-dependent proteolyis;

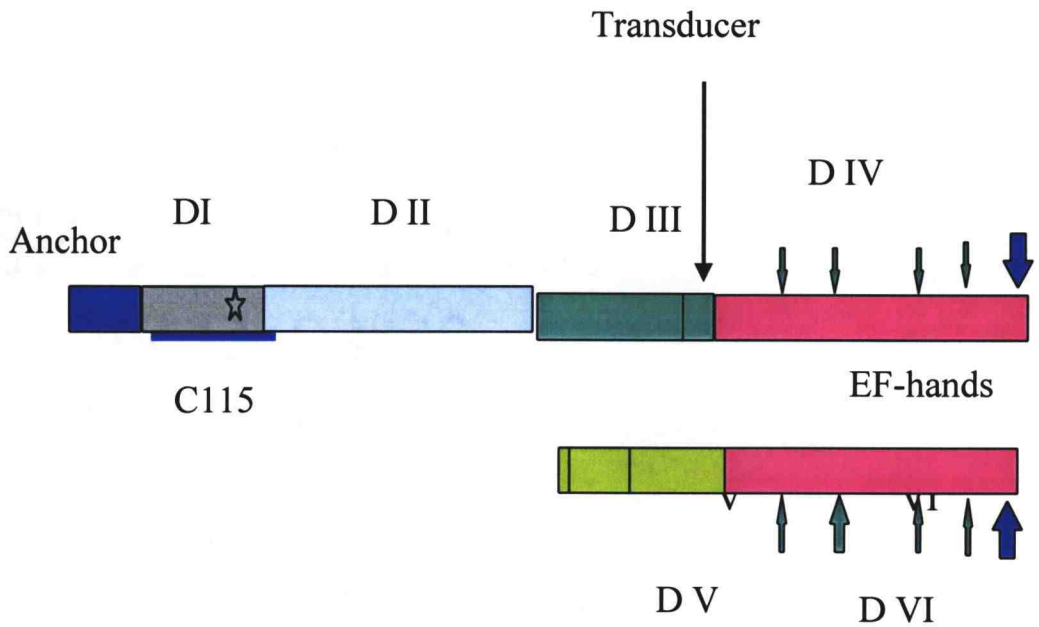

Figure 1.1. Schematic diagram of calpain large subunit and 30K small subunit showing the relevant domain positions. D I to D VI show the Domains from I to IV in the large subunit and the Domains V and VI in 30K small subunit. Short arrows pointed to the EF-hand structure in D IV and D VI. The long, large arrow points to the transducer region in D III and the star shape shows the position of cysteine (115) in D I.

b) Over-expression of antisense u-calpain. This construct was expected to reduce u-calpain activity.

c) Over-expression of antisense 30K subunit. This was expected to reduce both u- and m-calpain activities.

d) Over-expression of a fused antisense u-calpain+30K. similar to "c", this was expected to reduce both u- and m-calpain activities.

The Inducible Skeletal Muscle-Specific Expression System

The ecdysone-inducible mammalian expression system (Invitrogen, CA) is a regulated system available for inducible mammalian expression. It has greater than 200-fold inducibility in mammalian cells and almost no detectable basal expression (81). This system includes two vectors: one is expression vector pIND in which the gene of interested is subcloned. The other is regulatory vector pVgRXR, which ubiquitously expresses ecdysone receptor EcR and RXR. With the help from inducer ecdysone or its analogue ponasterone A the two receptors EcR and RXR form a heterodimer which binds to a promoter and initiates transcription of the gene of interest subcloned in pIND.

RXR is ubiquitously expressed in mammalian cells. Hence, only expression of EcR is adjustable for tissue specific expression. Because we were interested in the down-regulation of u-calpain in skeletal muscle specifically, the skeletal muscle α-actin promoter was used to control the expression of EcR. The cytomegalovirus promoter (pCMV), which controls the expression of EcR, was therefore replaced with the α-actin promoter. The modified vector pVgRXR was called pAP (α-**a**ctin **p**romoter). Then, the original ecdysone inducible **ubiquitous** mammalian expression system became an ecdysone inducible **skeletal muscle-specific** expression system. The new system opens a new avenue for researchers to more precisely control expression of genes specifically in skeletal muscle.

References

1. Sato, N., Murata, K., Watanabe, K., Hayami, T., Kariya, Y., Sakaguchi, M., Kimura, S., Nonaka, M., and Kimura, A. (1988) Growth-promoting activity of tuna growth hormone and expression of tuna growth hormone cDNA in *Escherichia coli*. Biotech. Appli. Biochem. 10:385-93.

2. Vasilatos-Younken, R., Zhou, Y., Wang, X., McMurtry J.P., Rosebrough, R.W., Decuypere, E., Buys, N., Darras, V.M., Van der Geyten, S., and Tomas, F. (2000) Altered chicken thyroid hormone metabolism with chronic GH enhancement *in vivo*: consequences for skeletal muscle growth. J. Endocri. 166:609-20.

3. Yee, S.P., and Rigby, P.W. (1993) The regulation of myogenin gene expression during the embryonic development of the mouse. Genes Dev. 7:1277-89.

4. McPherron, A.C., and Lee, S.J. (1997) Regulation of skeletal muscle mass in mice by a new TGF-beta super family member. Nature 387:83-90.

5. Dambadur, R., Sharma, M., Smith, T.P., and Bass, J.J. Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle. Genome Res. 1997 7:910-6.

6. McPherron, A.C., and Lee, S.J. (1997) Double muscling in cattle due to mutations in the myostatin gene. Proc. Natl. Acad. Sci. USA. 94:2457-61.

7. Grobet, L., Martin L.J., Poncelet, D., Pirottin, D., Brouwers, B., Riquet, J., Schoeberlein, A., Dunner, s., Menissier, F., Massabanda, J., Fries, R., Hanset, R., and Georges, M. (1997) A deletion in the bovine myostatin gene causes the double-muscled phenotype in cattle. Nat. Genet. 17(1):71-4.

8. Low, R.B. and Goldberg, A.L. (1973) Non-uniform rates of turnover of myofibrillar proteins in rat diaphragm. J. Cell Biol. 56: 590-5.

9. 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 253:192-3.

10. Goldberg, A.L. (1969a) Protein turnover in skeletal muscle. I. Protein catabolism during work-induced hypertrophy and growth induced with growth hormone. J. Biol. Chem. 244:3217-22.

11. Goldberg, A.L. (1969b) Protein turnover in skeletal muscle. II. Effects of denervation and cortisone on protein catabolism in skeletal muscles. J. Biol.Chem. 244:3223-9.

12. Huang, J., and Forsberg, N.E. (1999) Role of calpain in skeletal-muscle protein degradation. Proc. Natl. Acad. Sci. USA. 95:12100-5.

13. Nishiura, I., Tanaka, K., Yamato, S., and Murachi, T. (1978) The occurrence of an inhibitor of Ca^{2+}-dependent neutral protease in rat liver. J. Biochem. 84:1657-9.

14. Waxman, L., and Krebs, E.g. (1978) Identification of two protenase inhibitors from bovine cardiac muscle. J. Biol. Chem. 253:5888-91.

15. Ono, Y., Sorimachi, H. and Suzuki, K. (1999) The calpain superfamily. In *Calpain--Pharmacology and Toxicology of Calcium-Dependent Protease.* edited by Wang K.K.W. and Yuen, P.-W. 1--23.

16. Sorimachi, H., Ishiura, S., and Suzuki, K. (1997) Structure and physiological function of calpains. Biochem. J. 328:721-32.

17. Denison, S. H., Orejas, M., and Arst, H.N., Jr. (1995) Signaling of ambient pH in *Aspergillus* involves a cysteine protease. J. Biol. Chem. 270:28519-22.

18. Patrick, G.N., Zukerberg, H., Nikolic, M., de la Monte, S., Dikkes, P., and Tsai, L.H., (1999) Neurotoxicity induces cleavage of p35 to p25 by calpain. Nature 402:625-22.

19. Arthur, J.S.C., Elce, J.S., Hegadorn, C., Williams, K., and Greer, P.A. (2000) Disruption of the murine calpain small subunit gene, Capn4: calpain is essential for embryonic development but not for cell growth and division. Molec. Cell. Bio. 20:4474-81.

20. Richard, I., Broux, O., Allamand, V., Fougerousse, F., Chiannilkulchai, N., Bourg, N., Brenguier, L., Devaud, C., Pasturaud, P., and Roudaut, C., (1995) Mutations in the proteolytic enzyme calpain 3 cause limb-gridle muscular dystrophy type 2a. Cell 81:27-40.

21. Yoshimura, N., Kikuchi, T., Sasaki, T., Kitahara, A., Hatanaka, M., and Murachi, T. (1983) Two distinct Ca^{2+}proteases (calpain I and calpain II) purified concurrently by the same method from rat kidney. J. Biol. Chem. 258:8883-9.

22. Sormachi, H., Kmajoh-Ohmi, L., Emori, Y., Kawasaki, H., Ohno, S., Minami, Y., and Suzuki, K. (1989) Molecular cloning of a novel mammalian calcium-dependent protease distinct form both m- and u-types. J. Biol. Chem. 264:20106-11.

23. Ma, H., Fukiage, C., Azuma, M., and Shearer, T.R. (1998) Molecular cloning and expression of calpain Lp82 from rat lens: A splice variant of muscle p94 mRNA with insert regions deleted. Investigat. Ophthalmol.Vis. Sci. 39:454-61.

24. Ma, H., Hata, I., Fukiage, C., Azuma, M., and Shearer, T.R. (2000) Lp85 calpain is an enzymatically active rodent-specific isozyme of lens Lp82. Curr. Eye Res. 20:183-9.

25. 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 Ca(2+)-binding domain. J. Biol. Chem. 268:19476-82.

26. Lee, H.J., Sorimachi, H., Jeong, S.Y., Ishiura, S., and Suzuki, K. (1998) Molecular cloning and characterizatin of a novel tissue-specific calpain predominantly expressed in the digestive tract. Biol. Chem. 379:175-83.

27. Dear, N., Matena, K., Vingron, M., and Boehm, T. (1997) A new subfamily of vertebrate calpains lacking a calmodulin-like domain: implications for calpain regulation and evolution. Genomics 45:175-84.

28. Delaney, S.J., Hayward, K.C., Barleben, F., Fischbach, K.F., and Gabor Miklos, G.L. (1991) Molecular cloning and analysis of small optic lobes, a structural brain gene of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA. 88:7213-18.

29. Emori, Y., and Saigo, K. (1994) Calpain localization changes during early embryonic development of Drosophila. J. Biol. Chem. 269:25173-42.

30. Waterson, R., Martin, C., Craxton, M., Huynh, C., Coulson, A., Hillier, L., Durbin, R., Green, P., Shown-keen, R., Halloran, N., Metzstein, M., Hawkins, T., Wilson, r., Berks, M., Du, Z., Thomas, K., ThierryMieg, J., and Sulston, J. (1992) A survey of expressed genes in *Caenorhabditis elegans*. Nature Genetics 1:114-23.

31. Wilson, R., Alinscough, R. et al. (1994) 2.2 Mb of contiguous nucleotide sequence from chromosome III of *C. elegans*. Nature 368:32-8.

32. Goll, K.E., Thompson, VF., Taylor, R.G., and Zalewska, T. (1992) Is calpain activity regulated by membranes and autolysis or by calcium and calpastatin? BioEssays 14:549-56.

33. Saido, T.C., Sorimachi, H., and Suzuki, K. (1994) Calpain: new perspectives in molecular diversity and physiological-pathological involvement. FASEB J. 8:814-22.

34. Yoshizawa, T., Sorimachi, H., Tomioka, S., Ishiura, S., and Suzuki, K. (1995) A catalytic subuint of calpain possesses full proteolytic activity. FEBS Letters 358:101-3.

35. Elce, J.S., Hedadorn, C., and Arthur, J.S.C. (1997) Autolysis, Ca^{2+} requirement, and heterodimer stability in m-calpain. J. Biol. Chem. 272:11268-75.

36. Hosfield, C.M., Elce, J.S., Davies, P.L., and Jia, Z. (1999) Crystal structure of calpain reveals the structural basis for Ca(2+)-dependent protease activity and a novel mode of enzyme activation. EMBO J. 18:6880-9.

37. Strobl S., Fernandez-Catalan C., Braun M., Huber R., Masumoto H., Nakagawa K., Irie A., Sorimachi H., Bourenkow G., Bartunik H., Suzuki K., and Bode W. (2000) The crystal structure of calcium-free human m-calpain suggests an electrostatic switch mechanism for activation by calcium. Proc. Nat. Acade. Sci. USA. 97:588-92.

38. Blanchard H, Grochulski P, Li Y, Arthur J.S., Davies P.L, Elce J.S, and Cygler M. (1997) Structure of a calpain Ca(2+)-binding domain reveals a novel EF-hand and Ca(2+)-induced conformational changes. Nature Struct. Biol. 4:532-8.

39. Lin G.D., Chattopadhyay D., Maki M., Wang 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. Nature Struct Biol. 4:539-47.

40. Yoshizawa T, Sorimachi H, Tomioka S, Ishiura S, and Suzuki K. (1995) Calpain dissociates into subunits in the presence of calcium ions. Biochem. Biophys. Res. Commun. 208:376-83.

41. Zhang W., and Mellgren R.L. (1996) Calpain subunits remain associated during catalysis. Biochem. Biophys. Res. Commun. 227:890-6.

42. Elce J.S., Davies P.L., Hegadorn C, Maurice D.H., and Arthur J.S. (1997) The effects of truncations of the small subunit on m-calpain activity and heterodimer formation. Biochem. J. 326:31-8.

43. Johnson, G.V., and Guttmann R.P. (1997) Calpains: intact and active? Bioessays 19:1011-8.

44. Saido TC, Nagao S, Shiramine M, Tsukaguchi M, Yoshizawa T, Sorimachi H, Ito H, Tsuchiya T, Kawashima S, and Suzuki K. (1994) Distinct kinetics of subunit autolysis in mammalian m-calpain activation. FEBS Lett. 346:263-7.

45. Tompa P, Baki A, Schad E, and Friedrich P. (1996) The calpain cascade. Mu-calpain activates m-calpain. J. Biol. Chem. 271:33161-4.

46. Sultan, K.R., Dittrich, B.T., and Pette, D. (2000) Calpain activity in fast, slow, transforming, and regenerating skeletal muscles of rat. Am. J. Physiol. 279:C639-47.

47. Thompson, V.F., Lawson, K., and Goll, D.E. (2000) Effect of u-calpain on m-calpain. Biochem. Biophys. Res. Commun. 267:495-9.

48. Melloni E., Averna M., Salamino F., Sparatore B., Minafra R., and Pontremoli S. (2000) Acyl-CoA-binding protein is a potent m-calpain activator. J. Biol. Chem. 275:82-6.

49. 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. 249:583-8.

50. Richard I., Broux O., Allamand V., Fougerousse F., Chiannilkulchai N., Bourg N., Brenguier L., Devaud C., Pasturaud P., and Roudaut C, et al. (1995) Mutations in the proteolytic enzyme calpain 3 cause limb-girdle muscular dystrophy type 2A. Cell 81:27-40.

51. Tagawa K., Taya C., Hayashi Y., Nakagawa M., Ono Y., Fukuda R., Karasuyama H., Toyama-Sorimachi N., Katsui Y., Hata S., Ishiura S., Nonaka I., Seyama Y., Arahata K., Yonekawa H., Sorimachi H., and Suzuki K.. (2000) Myopathy phenotype of transgenic mice expressing active site-mutated inactive p94 skeletal muscle-specific calpain, the gene product responsible for limb girdle muscular dystrophy type 2A.Hum. Mol. Genet. 9:1393-402.

52. Yoshikawa Y, Mukai H, Hino F, Asada K, Kato I. (2000) Isolation of two novel genes, down-regulated in gastric cancer. Jpn. J. Cancer Res. 91:459-63.

53. Liu K., Li L., Cohen S.N. (2000) Antisense RNA-mediated deficiency of the calpain protease, nCL-4, in NIH3T3 cells is associated with neoplastic transformation and tumorigenesis. J. Biol. Chem. 275:31093-8.

54. Zimmerman U.J., Boring L., Pak J.H., Mukerjee N., and Wang K.K.. (2000) The calpain small subunit gene is essential: its inactivation results in embryonic lethality. IUBMB Life. 50:63-8.

55. Saatman K.E., Zhang C., Bartus R.T., and McIntosh T.K. (2000) Behavioral efficacy of posttraumatic calpain inhibition is not accompanied by reduced spectrin proteolysis, cortical lesion, or apoptosis. J. Cereb. Blood Flow Metab. 20:66-73.

56. Shields D.C., Schaecher K.E., Hogan E.L., and Banik N.L. (2000) Calpain activity and expression increased in activated glial and inflammatory cells in penumbra of spinal cord injury lesion. J. Neurosci. Res. 61:146-50.

57. Saatman K.E., Murai H., Bartus R.T., Smith D.H., Hayward N.J., Perri B.R., and McIntosh T.K. (1996) Calpain inhibitor AK295 attenuates motor and cognitive deficits following experimental brain injury in the rat. Proc. Natl. Acad. Sci. USA. 93:3428-33.

58. Ray S.K., Matzelle D.C., Wilford G.G., Hogan E.L., Banik N.L.. (2000) E-64-d prevents both calpain upregulation and apoptosis in the lesion and penumbra following spinal cord injury in rats. Brain Res. 867:80-9.

59. Ray S.K., Fidan M., Nowak M.W., Wilford G.G., Hogan E.L., and Banik N.L. (2000) Oxidative stress and Ca2+ influx upregulate calpain and induce apoptosis in PC12 cells. Brain Res. 852:326-34.

60. Maruyama K., Usami M., Kametani F., Tomita T., Iwatsubo T., Saido T.C., Mori H., and Ishiura S. (2000) Molecular interactions between presenilin and calpain: inhibition of m-calpain protease activity by presenilin-1, 2 and cleavage of presenilin-1 by m-, mu-calpain. Int. J. Mol. Med. 5:269-73.

61. Nath R., Davis M., Probert A.W., Kupina N.C., Ren X., Schielke G.P., and Wang K.K. (2000) Processing of cdk5 activator p35 to its truncated form (p25) by calpain in acutely injured neuronal cells. Biochem. Biophys. Res. Commun. 274:16-21.

62. Lee M.S., Kwon Y.T., Li M., Peng J., Friedlander R.M., and Tsai L.H. (2000) Neurotoxicity induces cleavage of p35 to p25 by calpain. Nature 405:360-4.

63. Kusakawa G., Saito T., Onuki R., Ishiguro K., Kishimoto T., and Hisanaga S. (2000) Calpain-dependent proteolytic cleavage of the p35 cyclin-dependent kinase 5 activator to p25. J. Biol. Chem. 275:17166-72.

64. Touyarot K., Poussard S., Verret C., Aragon B., Cottin P., Nogues X., and Micheau J. (2000) Calpain-PKC inter-relations in mouse hippocampus: a biochemical approach. Neurochem. Res. 25:781-90.

65. Atencio I.A., Ramachandra M., Shabram P., and Demers G.W. (2000) Calpain inhibitor 1 activates p53-dependent apoptosis in tumor cell lines. Cell Growth Differ. 11:247-53.

66. Ray S.K., Matzelle D.C., Wilford G.G., Hogan E.L., and Banik N.L. (2000) E-64-d prevents both calpain upregulation and apoptosis in the lesion and penumbra following spinal cord injury in rats. Brain Res. 867:80-9.

67. Zhu D.M. andUckun F.M. (2000) Calpain inhibitor II induces caspase-dependent apoptosis in human acute lymphoblastic leukemia and non-Hodgkin's lymphoma cells as well as some solid tumor cells. Clin. Cancer Res. 6:2456-63.

68. Rami A., Agarwal R., Botez G., and Winckler J. (2000) mu-Calpain activation, DNA fragmentation, and synergistic effects of caspase and calpain inhibitors in protecting hippocampal neurons from ischemic damage. Brain Res. 866:299-312.

69. Lankiewicz S., Marc Luetjens C., Truc Bui N., Krohn A.J., Poppe M., Cole G.M., Saido T.C., and Prehn J.H. (2000) Activation of calpain I converts excitotoxic neuron death into a caspase-independent cell death. J. Biol. Chem. 275:17064-71.

70. Barnes T.M., and Hodgkin J. (1996) The tra-3 sex determination gene of Caenorhabditis elegans encodes a member of the calpain regulatory protease family. EMBO J. 15:4477-84.

71. Nelson W.J, and Traub P. (1983) Proteolysis of vimentin and desmin by the Ca2+-activated proteinase specific for these intermediate filament proteins. Mol. Cell. Biol. 3:1146-56.

72. Dayton, W.R., Goll, D.E., Stromer, M.H., Reville, W.J., Zeece, M.g., and Robson, R.M. (1975) Some properties of a Ca2+-activated protease that may be involved in myofibrillar protein turnover. In *Cold Spring Harbor Conferences on Cell Proliferation*, vol. 2, *Proteases and Biological Control*, eds. Reich, E., Rifkin, D.B., and Shaw E., 551-7. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

73. Goll D.E., Thompson V.F., Taylor R.G., and Christiansen J.A. (1992) Role of the calpain system in muscle growth. Biochimie. 74:225-37. Review.

74. Spencer M.J., and Tidball J.G. (1992) Calpain concentration is elevated although net calcium-dependent proteolysis is suppressed in dystrophin-deficient muscle. Exp. Cell Res. 203:107-14.

75. Goll D.E., Dayton W.R., Singh I., and Robson R.M. (1991) Studies of the alpha-actinin/actin interaction in the Z-disk by using calpain. J. Biol. Chem. 266:8501-10.

76. Groll M., Ditzel L., Lowe J., Stock D., Bochtler M., Bartunik H.D., and Huber R. (1997) Structure of 20S proteasome from yeast at 2.4 A resolution. Nature 386:463-71.

77. 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. 271:26690-7.

78. Forsberg N.E., Ilian M.A., Ali-Bar A., Cheeke 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. 67:3313-21.

79. Bardsley R.G., Allcock S.M., Dawson J.M., Dumelow N.W., Higgins J.A., Lasslett Y.V., Lockley A.K., Parr T., and Buttery P.J. (1992) Effect of beta-agonists on expression of calpain and calpastatin activity in skeletal muscle. Biochimie. 74:267-73.

80. Parr T., Bardsley R.G., Gilmour R.S., and Buttery P.J. (1992) Changes in calpain and calpastatin mRNA induced by beta-adrenergic stimulation of bovine skeletal muscle. Eur. J. Biochem. 208:333-9.

81. No D., Yao T.P., and Evans R.M. (1996) Ecdysone-inducible gene expression in mammalian cells and transgenic mice. Proc. Natl. Acad. Sci. USA. 93:3346-51.

Chapter II

Regulation of *in vitro* Skeletal Muscle Protein Degradation By μ-Calpain in Rat

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Abstract

Ubiquitous calcium-dependent cysteine protease (calpains) play broad physiological and pathological roles in life. There is considerable evidence showing that ubiquitous calpains are the rate-limiting factors for animal skeletal muscle growth. Hence, the first goal of this thesis was to understand the role of u-calpain in skeletal muscle protein degradation in cultured muscle cells. Several strategies were developed to down-regulate endogenous u-calpain activity and m-calpain activity in rat myotubes. These included over-expression of antisense u-calpain (AnsL), dominant negative u-calpain (DN-u-CL), antisense 30K subunit (AnsS) and fused antisense u-CL/30K (AnsLS, i.e., 80K/30K). These constructs were expressed in stable rat L8 myotubes and their effects on a variety of targets were examined. The ability to regulate calpain activity was confirmed by fodrin degradation (an index of calpain activity). Our data supported the contention that u-calpain contributes significantly to total protein degradation in myotubes. Specifically, over-expressing DN-u-calpain reduced total protein degradation by 7.9% ($P<0.01$) at 24 hr time point and by 10.6% ($P<0.01$) at a 48 hr time point. Similarly, over-expression of antisense u-calpain and the 30K subunit reduced total protein degradation significantly at the 24 hr time point ($P<0.05$). However, over-expression of the fused antisense (80K/30K) did not affect ($P>0.05$) total protein degradation. In addition to this we determined that desmin was a calpain substrate and that calpain could not degrade tropomyosin.

The second goal of this study was to evaluate the relationships among u- and m-calpain and the 30K subunit. The rationale for this study was that our earlier work indicated coordinated regulation of the calpain subunits. Our cell lines afforded us the opportunity to study these relationships in detail. In this study, we evaluated effects of down-regulating u-calpain and 30KD subunit on expression of all subunits. Our data demonstrated for the first time that the transcription and translation of u-calpain and 30K, and m-calpain and 30K are coordinately regulated, respectively. However, the expression of u-calpain did not affect the expression of m-calpain. Specifically, a reduction in 30K

expression increased u- and m-calpain expression at both mRNA and protein levels. Over-expression of DN-u-CL increased 30K expression at both mRNA and protein levels.

Keywords: Ecdysone expression system, skeletal muscle, u-calpain, dominant negative, antisense, desmin, tropomyosin

Introduction

The calpains (EC3.4.22.17) are a super-family of calcium-dependent cytosolic non-lysosomal cysteine proteinases first discovered 25 years ago (1). Because of their diversities in expression pattern, protein composition and dependency on calcium concentration for their half-maximal activities, the members of this super-family show extremely broad physiological and pathological functions in various organisms from fungi to human (2-6). According to their structure, the calpains are classified as conventional calpains and atypical calpains.

The conventional calpains include u-calpain (micro- or calpain I), m-calpain (milli- or calpain II), and regulatory subunit 30K or calpain-4 (7). m-Calpain and u-calpain form heterodimers with the 30k small subunit. Both m- and u-calpain large subunits may be divided into four domains from the N-terminal: a propeptide domain (I), a protease domain (II), a regulatory domain (III), and a calmodulin-like calcium-binding domain (IV). The 30K subunit can be divided into an N-terminal glycine-rich domain (V) and calmodulin-like calcium binding-domain (VI). Some other conventional calpains might not form a heterodimer with 30K and are expressed tissue-specifically. These include skeletal muscle-specific p94 (22), the lens-specific splicing variants of p94 (Lp82 and Lp85) (23, 24), stomach-specific calpains nCL-2 and -2' (25), digestive organ-specific calpain nCL-4 (26), placenta-specific species CAPN6 (27), sol and Dm-calpain (28, 29) from *D. melanogaster,* p71, p72 and p92 from *C. elegans* (30, 31).

Calpains exist in the cytosol as inactive proenzymes. *In vitro* activation of u- and m-calpains requires ~10-50 uM and ~300-500 uM, respectively. These concentration of Ca^{2+} are considerably higher than physiological Ca^{2+} concentrations (generally <1 uM) (32). Hence, *in vivo*, calpains must be sensitized to Ca^{2+} and be activated before they mediate their physiological roles. Three factors have been established as potential regulators of Ca^{2+} sensitivity of calpains: autolysis, phospholipids and the 30K subunit (33).

The 30K subunit functions like a chaperone which stabilizes the large subunits (u- and m) *in vivo* and *in vitro* (34, 35). Without binding with 30K, the intact or autolyzed large subunits are quickly degraded. Purification of large subunits as monomers from biological sources has never been successful. Based on the X-ray structure of rat (36) and human (37) m-calpain in the absence of Ca^{2+}, plus information from the crystal structure of the Ca^{2+}-binding 30K homodimer (38, 39), the mystery of calpain activation has finally been clarified. Without Ca^{2+} binding, m-calpain binds with 30K subunit. However, the active site in Domain I and the active sites in the Domain II are kept away from each other and thereby prevented from forming an active site cleft. The N-terminal anchor (part of Domain I) is an α-helix that tethers Domain I to Domain VI in the 30KD regulatory subunit and plays a key role in the activation process. Ca^{2+}-binding causes conformational changes in Domain IV and Domain VI, which may allow the transducer (in Domain III) to release constraints on other domains and exert more flexibility to Domain II. This might cause the movement of Domain II to Domain III. Spontaneously, the release of the anchor yields a more flexible Domain I. The relief of the restraints from Domains-I and -II allows formation of the active site cleft at the interface of Domain-I and -II via the rotation of these domains toward each other. Following the formation of the active site, intermolecular autolysis of the N-terminal anchor activates calpain by permitting activity at lower Ca^{2+}-concentration. The C_2-like Domain III may be responsible for promoting binding of active calpain to the membrane in response to Ca^{2+}, thereby relieving inhibition from calpastatin and promoting digestion of calpain substrates (36). This activation mechanism might help to resolve the following seemingly contradictory observations: the dissociation of 30K from the large subunit in the presence of Ca^{2+} (for the activation of large subunits) and that calpain remains as a heterodimer during catalysis (for stabilizing the conformation of large subunits and avoiding from the quick degradation) (40, 41,42).

Despite recent knowledge concerning calpain activation conflicts still exist regarding the activation mechanisms and functions for both m- and u-calpains. Other reports found out that proteolysis by m-calpain occurred without large subunit autolysis (35, 43, 44). In

order to achieve activation in the presence of low physiological Ca^{2+} concentrations, some reports suggested that activated u-calpain can activate m-calpain (45, 46). Not surprisingly, others have not substantiated these reports (47). In addition, there may be specific activators which sensitize m- and u-calpains to Ca^{2+}. For example, UK114 modulates u-calpain (48) and acyl-CoA-binding protein alters Ca^{2+}-sensitivity of m-calpain (49).

The physiological and pathological roles of calpains are not fully established. But recent studies are providing new insights, particularly for the tissue-specific calpains. Calpain-3, or p94, has drawn broad attention since the discovery that defects in the p94 gene causing loss of p94 activity lead to the development of Limb-girdle Muscular Dystrophy Type-2A (50). Studies with p94 knock-out mice provided similar conclusions (51). Two research groups recently independently identified deficiency of gastric-specific calpain (nCL4) and reported that it related to carcinogenesis (52, 53). Len-specific calpains Lp82 and Lp85 participate in cataractogenesis (23, 24). The *Caenorhabditis elegans* calpain homologue (Tra3) determines female sexual development (70).

Compared to the tissue-specific calpains, roles of the house keeping calpains are not fully known. Most information concerning functions is based on inhibitors but recent genetic approaches have being developed. m- and u-calpain and 30K subunit are ubiquitously expressed, so, their function should be much broader than their tissue-specific counterparts. The most significant report concerning the function of 30K relates to its lethality at mid-gestation in 30K-knockout (K/O) mice embyros (5,54). The 30K K/O embryos, which died at mid-gestation, showed defects in the cardiovascular system, hemorrhaging, and accumulation of erythroid progenitors (5). Even though there is no way to identify specific functions of 30K in K/O adult mice, the lethal embryos tell us that the function of 30K is highly significant. Both u- and m-calpain activities are abolished in 30K K/O embryos (5). This deletion, however, didn't affect survival and proliferation of cultured embryonic stem cells or embryonic fibroblasts, even in early stages of organogenesis. The serious consequences to the 30K K/O are likely based on the spontaneous deletion of both activities of u- and m-calpains. Hence, the subsequent

double deletion of u- and m-calpains contributed to the embryonic developmental defects in 30K K/O, rather than from direct effects of the deletion of the 30K subunit. Besides its chaperon-like function in stabilizing u- and m-calpains, at this stage there is no direct physiological or pathological function assigned to the 30K subunit.

From knowledge of the 30K K/O defects in embryonic development, we may expect to see a wide range of functions for u- and m-calpains. The substrate specificity for u- and m-calpains is variable. Because the activity of calpains is dependent on the concentration of Ca^{2+}, any physiological condition which disturbs Ca^{2+} homeostasis (increase or decrease) may change calpain activity. Similarly, altered transcription and translation of calpains will change their activity, too. In spinal cord injury and traumatic brain injury, influx of Ca^{2+} into the lesion increases calpain activity. Excessive active calpain digests cytoskeletal proteins (e.g., fodrin) and neurofilament proteins (NFP) (56, 57, 58). Oxidative stress in central neuronal cells had a similar effect (59). Calpain inhibitors, (e.g., E-64-D and AK295) can prevent neural damage by inhibiting calpain activity in spinal cord injury and traumatic brain injury. There is also an opposing report stating that behavioral efficacy of post-traumatic calpain inhibition is not accompanied by reduced spectrin proteolysis, cortical lesion, or apoptosis (55). Calpain might initiate pathogenesis of Alzheimers' disease (60, 61, 62, 63) by its relationship with p35, and it also might play a role in learning and memory (64).

The possibility that calpain plays a role in apoptosis is very important because apoptosis is so critical to many cellular events including normal development, cancer and aging. However, reported functions of u- and m-calpain in apoptosis are not consistent. u-Calpain might play more important role than m-calpain in apoptosis (68,69). Calpain can degrade p53. This means that calpain can prevent wild-type p53 cells, but not mutated or null p53 cells, from entering apoptosis (65). E-64-d can prevent both calpain up-regulation and apoptosis in the lesion and penumbra following spinal cord injury in rats (66). However, calpain inhibitor II (CPI-2) induces caspase-dependent apoptosis in acute lymphoblastic leukemia and non-Hodgkin's lymphoma as well as in some solid tumor cells (67). Calpain inhibitor I (CPI-1) can activate p53-dependent apoptosis (65). The

discrepancy among these reports might be explained by the differences between experimental systems (i.e., spinal cord injury versus tumor cell lines). Alternatively, discrepancy may be caused by the side effects of different calpain inhibitors.

As indicated, most studies of calpain function have relied on calpain inhibitors. In these types of studies, the specificity of the inhibitor for calpains should always be a concern. Reversible calpain inhibitors, (e.g., calpain inhibitors I and II, AK295) (8, 9), form hemithioacetal or ketal structures with the active-site cysteine to inactivate calpain. Irreversible calpain inhibitors, (e.g., the E64 family) (10), can irreversibly alkylate the active-site cysteine thiol to form a sulfide. Recently, more potent reversible inhibitors (e.g., PD150606), which target the calcium-binding domain of calpain, have been developed. Many of these have high specificity for calpains (11, 12). However, most of these inhibitors also inhibit other cysteine proteases and, in some cases, even serine and threonine proteases. Because present calpain isoform-specific inhibitors have not been developed, understanding functions of individual calpain depends on genetic approaches.

The physiological functions of u- and m-calpain in the metabolic turnover of myofibrillar proteins is more interesting for animal scientists. Muscle proteins turn over with half-lives ranging from 2 to 10 days (15) or 5 to 10% per day. And, it has been estimated that 15 to 25% of the food ingested by animals is used to replace the metabolically turned-over muscle protein (16). According to the physiological condition of animals, this rate could be much higher (17, 18).

Past research results pinpoint calpains to a rate-limiting role in muscle growth. Myofibrils in skeletal muscle are too big to be engulfed by the lysosome and the central cavity of the proteasome is too small for intact myofibrils to enter (76). Hence, myofibrils must first be detached from the sarcomere, then degraded by the proteasome (77). Calpains have very unique and limited specificity for digesting myofibrillar/cytoskeletal proteins, (e.g., desmin (71), troponin I and T (72), and spectrin; 19). Immunolocalization studies of skeletal muscle placed calpain and calpastatin on the Z-disk and the I-band (73, 74). The rate-limiting relationship between calpains and skeletal muscle growth is

further demonstrated by studies on the skeletal muscle growth-enhancement caused by β-adrenergic agonists: skeletal muscle calpastatin (endogenous inhibitor of the calpains) is increased significantly (78,79,80), but the activities of u- and m-calpain were not significantly affected (78, 81). Even more direct evidence for a role of calpains in myofibrillar protein degradation comes from our lab (19). Over-expression of calpastatin inhibitory domain (CID) in rat L8 myotubes reduced total skeletal muscle protein degradation by 63% and over-expression of active site-mutated m-calpain (dominant negative m-calpain) reduced total skeletal muscle protein degradation by 30%. Because expression of CID inhibits the activity of u- and m-calpains and because DN-m-calpain is expected to specifically inhibit m-calpain-dependent digestion, we estimate that, by difference, u-calpain accounts for reduction of muscle total protein degradation by 30%.

In this study, we developed cell lines in which we could evaluate specific functions of u-calpain. Two proteins we studied were **desmin** and **tropomyosin** and hypothesized that both were degraded by u-calpain. Desmin intermediate filament was reported to be a calpain substrate (13). However, it is not known which of the calpains degrades desmin. Desmin surrounds the Z disc and links adjacent myofibrils at the level of the Z disc. It also links myofibrils to the sarcolemma at the level of the costamere (14). Desmin is crucial for maintaining architectural and functional integrity of striated muscle (75). The desmin cytoskeleton is linked to muscle mitochondrial distribution and respiratory function (82). Desmin also accumulated in muscle diseases which are called desminopathies. The mechanism causing for their accumulation is not known. But it is essential to know this to understand the basic for the disease. Hence, we studied desmin degradation, in particular, for this purpose. In striated muscle, tropomyosin is a coiled coil protein that binds head-to-tail along the length of actin filaments. Tropomyosin plays the central role in regulation by moving its position relative to actin. It is not known if tropomyosin is a calpain substrate. Some reports indicated that beta-tropomyosin can be digested by calpain (83, 84), whereas other reports claimed that tropomyosin could not be degraded by calpains (85, 86). A goal of this work was to determine whether tropomyosin was a true substrate of u-calpain.

Based on the above rationale, we designed the following experiments to test the hypothesis that down-regulation of calpain will decrease skeletal muscle total protein degradation in rat L8 cell. To reduce calpain activity we used several strategies. These included:

a) Over-expression of dominant negative u-calpain. (this construct was expected to express a "dead" protease which would compete for substrate with endogenous u-calpain and thereby reduce u-calpain-dependent proteolyis),

b) Over-expression of antisense u-calpain. (This construct was expected to reduce u-calpain activity.)

c) Over-expression of antisense 30K subunit. (This was expected to reduce both u- and m-calpain activities.)

d) Over-expression of a fused antisense u-calpain+30K. (Similar to "c", this was expected to reduce both u- and m-calpain activities.)

We report here that our strategies worked very well for down-regulating the activities of both u- and m-calpain. To our knowledge, this is the first time scientists have been able to study relationships among u- and m-calpains and 30K subunit in vivo, rather than utilizing inhibitors. There is dynamic relationship, at least in myotubes, for the transcription and translation of u-CL, m-CL and 30K subunits. Under our experimental conditions, we found out that desmin is a true substrate of u-calpain. Contrary to previous reports, tropomyosin is not a substrate of calpain.

Materials and Methods

Plasmid Construction

Development of a skeletal muscle-specific inducible expression system: The ecdysone Mammalian Expression System (Invitrogen, Carlsbad, CA) is a ubiquitous expression system. In order to express the gene of interest in this system, specifically in skeletal muscle, we replaced the constitutivelly active cytomegalovirus promoter (pCMV), which controls the expression of ecdysone receptor, by skeletal muscle α-actin promoter (the new vector was designated pAP). Skeletal α-actin is controlled by mytogenesis factors and thereby was expected to confer expression in differentiated myotubes. The details about this construct and the functional testing of this construct are described in the Chapter II (Development of a Skeletal Muscle-Specific Ecdysone-Inducible Expression System).

In the process of preparing plasmid constructs, we often used a mixture-screening method for increasing the chance to pickup the recombinant plasmid. The details of mixture-screening are described in the Appendix (Mixture-Screening Method for Identifying Bacterial Colonies Containing the recombinant Plasmid DNA).

***Site-directed mutagenesis of rat μ-calpain*:** Full-length rat u-calpain large subunit (abbreviated as u-CL) cDNA, subcloned into pUC18 vector in the EcoRI site was a gift from Dr. Suzuki (University of Tokyo, Japan). Polymerase chain reaction (PCR)-based site-directed mutagenesis was developed. In brief, u-calpain was divided into two fragments: one fragment contained a mutated active site (Cys115→Ala) and the second fragment contained the remaining portion of u-calpain. The first fragment was digested by *Bam*HI (New England Biolabs, Beverly, MA), the second fragment was digested by *Eco*RI (New England Biolabs), and pIND vector (expression vector in the ecdysone system, Invitrogen) was double-digested by these two enzymes. After gel purification of these three fragments, they were ligated together in one ligation reaction (24 hr ligation, DNA T4 ligase, New England Biolabs). Ten ul of the ligated product were used to transform self-made 100 ul competent cells of *E. coli* DH5α strain. Mutated site and

orientation of the ligation were verified by sequencing (Central Services Lab, Center for Gene Research and Biotechnology, Oregon State University). This mutated u-calpain was designated as DN-u-calpain (i.e., dominant negative u-calpain).

In order to distinguish the endogenous u-calpain and the mutated DN-u-calpain, we marked the u-calpain-DN by 10 amino acids of Myc-tag at the N-terminal of DN-u-calpain. The Myc-tagged sequence was synthesized by the Central Services Lab (OSU). The Myc-tagged DN-u-calpain (see below) was constructed as follows: 5'-restriction site----u-calpain native Kozak sequence----start codon----Myc----(5'-sequence of u-calpain)----DN-u-calpain----restriction site. The plasmid containing the Myc-tag was first verified by PCR (the forward primer is 5'--GGATGGAGCAAAAACTAATCAGC--3', the reverse primer is 5'--GCGCGAATTCAGGAATACATAGTCAGCTG--3'), then the orientation, Myc-tag sequence and reading frame were verified by sequencing.

The simplified Myc-DN sequence order is as follows:

5'--**GG TAC C** *CCAGG* **ATG** GAG CAA AAA CTA ATC AGC GAG GAG GAC CTA

KpnI site Kozak |←----------Myc tag----------→|

GC**G GAT CC** G CAG CAA TCT CCC AAA CTC CCC TTC C*CC AGG* **ATG**--

BamHI site Kozak

DN-u-calpain---3'

Preparation of u-calpain Antisense cDNA: Full length u-calpain antisense cDNA was generated by PCR and subcloned into pIND (expression vector in Ecdysone Inducible Expression System, Invitrogen). The forward primer AnsL-F was 5'--GCGCGGATCCCGCTTCATGCAAAC--3' and the reverse primer AnsL-R was 5'--GCGCGAATTCCCCTTCCCCAGG--3'. The PCR reaction was completed in 100 ul volume with 2.5 mM $MgCl_2$, 2.5 mM dNTP mix, 10 ul *Taq* DNA polymerase buffer (10X), 50 ng of rat u-calpain cDNA, 2 ul primer mixture of AnsL-F and AnsL-R (25 pmole/ul each), 1 ul *Taq* DNA polymerase (5 U/ul, Promega, Madison, WI) and water. The thermocycler conditions were set as follows: 94°C for 4 min, 30 cycles of 94°C for

30 sec, 58°C for 30 sec and 72°C for 2 min, with an extra extension time of 8 min at 72°C. The 2.1kb fragment was then subcloned into pIND. The AnsL orientation and sequence were confirmed by sequencing. The vector was designated pIND-AnsL.

Preparation of 30k Antisense cDNA: A 30kD subunit cDNA was generously provided by Dr. John Elce (Queen's University, Canada). This was provided subcloned into pACpET. Whole cDNA was used to generate the 30k antisense cDNA by PCR using primers AnsS-F (5'--GCGCGGATCCACTCAGGAATACATAGTC--3') and AnsS-R (5'--GCGCGAATTCTTGGTGAACTCGTTCTTG--3'). The PCR reaction was completed in 100 ul volume with 2.5 mM $MgCl_2$, 2.5 mM dNTP mix, 10 ul *Taq* DNA polymerase buffer (10X), 50 ng of rat 30k cDNA, 2 ul primer mixture of AnsS-F and AnsS-R (25 pmole/ul each), 1ul Taq DNA polymerase (5 U/ul, Promega) and water. The thermocycler conditions were set as follows: 94°C for 4 min, 30 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min, with an extra extension time of 5 min at 72°C. The 810bp fragment was then subcloned into pIND. The AnsS (S means small subunit, i.e., 30k) orientation and sequence were confirmed by sequencing. The vector was designated pIND-AnsS.

Construct of fused u-CL and 30k Antisense cDNA: 30k antisense cDNA was generated via PCR (the forward primer was 5'--GCGCGAATTCAGGAATACATAGTCAGCTG--3' and the reverse primer was 5'--GCGCTCTAGATGTTCTTGGTGAACTCGTTC--3') and subcloned into the 3' end of u-CL antisense cDNA in pIND-AnsL. The region between the two antisense cDNAs was confirmed by sequencing. This plasmid was named as pIND-AnsLS (LS means large and small subunits).

Cell Culture System

Rat L8 myoblasts, and all other stable transfectants, were cultured in Dulbecco's Modified Eagle Medium (DMEM, low-glucose, Gibco, Gaithersburg, MD) with addition of 3.7g/L sodium bicarbonate, 100U/ml penicillin-streptomycin (Gibco) and 10% fetal bovine serum (FBS, Hyclone, Logan, UT). Final concentrations of G418 (Gibco) 400ug/ml and Zeocin (Invitrogen) 200 ug/ml were added to the FBS/DMEM medium for stable transfectants. When myoblast cells reached about 90% confluence, the DMEM/FBS medium was switched to DMEM with 2% horse serum (Hyclone) for inducing differentiation. Every two days the medium was replaced with fresh medium.

Trypsin 2.5 mg/ml was used to detach adherent cells from plates either for subcluture or long-term storage. FBS 10% medium was added to stop the trypsinization. Suspended cells were centrifuged at 1000 rpm for 4 min and the cell pellet was resuspended. For storage, the cells were placed in vials with a 9:1 ratio of FBS medium and DMSO (Sigma, St. Louis, MO) and stored at -80^{o}C. For subculture, the cells were diluted into appropriate volumes of FBS/DMEM medium and evenly distributed into plates.

Stable Cell Line Establishment

LipofectAMINE Reagent (Gibco) was used for the co-transfection of regulatory vector pAP (Figure 3.2) and the prospective expression vectors (one of pIND-DN, pIND-AnsS, pIND-AnsL, or pIND-AnsLS) into rat L8 myoblasts. Plasmid DNA was prepared by using a NucleoBond Plasmid Maxi Kit (Clontech, Palo Alto, CA) and the concentration was measured by comparing sample DNA with λ/HindIII DNA fragment standard (Gibco) on a 1% agarose TAE (40 mM Tris-acetate, 1 mM EDTA, and 1.14 mL/L glacial acetic acid) gel.

The transfection procedure was based on the manufacturer's guidelines with some modifications according to the character of rat myoblast L8 cell line. Briefly, the day

before transfection, we trypsinized 80% confluent myoblast cells on 100-mm plates and redistributed the cells onto three 100-mm plates so that they were more than 50% confluent the day of transfection. At the time of plating and during transfection, we avoided antibiotics. Immediately prior to the transfection, four ug (2ug of each of regulatory vector pAP and expression vector (pIND-DN, or pIND-AnsL, or pIND-AnsS, or pIND-AnsLS) were added to 300 ul serum-free medium. Similarly, 15 ul of LipofectAMINE reagent were diluted into 300ul serum-free medium. Then the diluted vector medium and diluted LipofectAMINE reagent were mixed and left at room temperature (RT) 15 minutes. While complexes were forming, we replaced medium on the cells with 5ml serum-free transfection medium. After this, we added the DNA-LipofectAMINE reagent complexes to the plates and mixed the medium gently. Cells were incubated at 37°C at 5% CO_2 for 3 hr. After 3 hr of incubation, we replaced the medium containing the complexes with fresh, complete medium and incubated the plates for one day. After 24 hr incubation, the medium in the plates was replaced by selection medium containing G418 (400ug/ml, Gibco) and Zeocin (200 ug/ml Gibco). After 24 hr incubation, cells on the plates were trypsinized and then the cells were redistributed in selection medium with dilution.

The co-transfected cell lines were named after their expression vector; namely Myc-DN (dominant negative cell line), AnsL (large subunit antisense cell line), AnsS (small subunit antisense cell line) and AnsLS (fused large and small subunit antisense cell line). Each cloned line was transferred from the original plate by a cloning ring when the clones were large enough to be visualized without a microscope. Selected clones were cultured sequentially in a 12-well plate, a 5-well plate, a 5-cm plate and a 10-cm plate. Ten to 15 clones of each stable cell line were selected. At least one vial of each clone in each cell line was stored in a -80°C freezer. Before checking the expression efficiency of the interested genes, all cell lines were checked for their differentiation capacity. Only the cell lines (i.e., clones) that showed good differentiation were used in further experiments.

Total RNA Extraction

The cell lines DN, AnsL, AnsS and AnsLS were cultured with selection medium until they reached 85 to 90% confluent. The medium was changed to differentiation medium (2% HS medium, HyClone). After differentiation, ponasterone A (10 ug/ml, an analogue of ecdysone, Invitrogen) was added to the treatment plates, or the same volume of 100% ethanol (vehicle) was added to the control plates. After 24 hr or 48 hr incubation, TRIzol Reagent (Gibco) was used to extract total RNA from the cells. The manufacturer's guidelines were followed with some modifications. Briefly, after residual culture medium was removed, and the monolayer of cells was directly lysed in the culture dish by adding 1 ml of TRIzol Reagent. The lysed cells were incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. After this, 0.2 ml of chloroform per 1ml of TRIzol Reagent was added and the tubes were shook vigorously by hand for 15 seconds. The tubes were allowed to stand at RT for 5 to 15 min then, centrifuged at 12,000Xg for 15 min at 4°C. After centrifugation, we transferred the upper aqueous phase to a new tube (the organic phase could be saved for isolation of total DNA and protein). Isopropyl alcohol 0.5 ml was used for the precipitation of RNA. To accomplish this, we incubated the tubes at RT for 10 min and centrifuged samples at 12,000 g for 8 min at 4°C. The supernatant was removed and the RNA pellet was washed with 1 ml of 75% ethanol. The sample was mixed by vortexing and centrifuged at 7,500Xg for 5 min at 4°C. At the end of the procedure, the RNA pellet was briefly air-dried, then dissolved in 30 ul of DEPC-treated water. Finally, we incubated RNA solution in a 57°C water bath for 10 min. RNA concentration was measured via spectrophotometer (DU.640B. Beckman, Palo Alto, CA) at $A_{260/280}$. The quality of RNA was determined by running 1 ug RNA on 1% TAE agarose gel. DNase treatment was used to treat RNA samples which were contaminated by genomic DNA.

Reverse Transcription-PCR (RT-PCR)

The Calypso RT-PCR System (Tetra Link International Inc. NY) is a one-step reaction system designed for sensitive, fast, reproducible and convenient analysis of RNA. The Calypsa RT-PCR system was utilized to check the expression of our genes of interest. The manufacturer's general protocols were followed with some modifications. Briefly, in multiple reactions, Master Mix 1 and 2 (from the protocol) were prepared separately. Twenty-five pmole of each forward and reverse primer were added and 500 ng RNA (in 100 ng/ul concentration) template were used. For all genes of interest, RT was performed at 50°C for 30 min and followed by inactivation of reverse transcriptase (94°C, 2 min). PCR was performed in 30 (for 30k) or 25 cycles. In the PCR process, the annealing temperature and the extension time for different templates were varied and detailed in the following text.

The PCR reaction for Myc-tagged DN-u-calpain PCR reaction was performed for 25 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 68°C for 1 min, with an extra extension period 5 min of 60°C. The forward primer Myc-seq was 5'--CTAATCAGCGAGGAGGACCTA--3', and the reverse primer u-CL-B-900 was 5'--TTGGCATCCGTCACAGAGTAT--3'.

The u-calpain PCR reaction was performed for 25 cycles at 94°C for 30 seconds, 58°C for 30 seconds, and 68°C for 1 min, with an extra extension period of 5 min at 68°C. The forward primer u-CL-F was 5'--ATGGCAGAGGAGTTAATCACA--3', and the reverse primer u-CL-B-900 was 5'--TTGGCATCCGTCACAGAGTAT--3'.

The small subunit 30K PCR reaction was performed for 30 cycles at 94°C for 30 seconds, 58°C for 30 seconds, and 68°C for 1 min, with an extra extension period for 5 min at 68°C. The forward primer 30k-Ans-C was 5'--CTTTGAGGCAGCAGGATTCCA --3', and the reverse primer u-30k-B was 5'--CAGCTGACTATGTATTCCTGA--3'.

The m-calpain PCR reaction was performed for 25 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 68°C for 1 min, with an extra extension period of 5 min at 68°C. The forward primer was 5'--AGAGAAGAAGGCTGACTACCA--3', and the reverse primer was 5'--TCTCAGGGTCCAGCTGCTTGA--3'.

The fused antisense AnsLS PCR was performed for 30 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 68°C for 1 min, with an extra extension period of 5 min at 68°C. The forward primer u-CL-B-900 was 5'--TTGGCATCCGTCACAGAGTAT--3' and the reverse primer 30k-Ans-C was 5'--CTTTGAGGCAGCAGGATTCCA--3'.

Ten ul of each RT-PCR reaction were used to check size of the RT-PCR products on a 2% agarose gel in TAE buffer.

Measurement of Total Protein Degradation

After two to three days of growth, the myotubes were about 90% confluent. The culture medium (10% FBS in DMEM) was changed to differentiation medium (2% Horse Serum in DMEM). Right before the cells began to differentiate, 0.5uCi/ml of ^{3}H-tyrosine (DuPont NEN, MA) were added to all plates for 24 hr. After differentiation was complete, all plates were washed twice with DMEM medium containing 2 mM cold tyrosine and refilled with HS medium (containing 2 mM tyrosine; chase). For the treatment plates, 10ug/ml ponasterone A (inducer) were added, whereas 5 ul/ml 100% ethanol (vehicle) were added to the control plates. After 20 min, 1 ml of the medium was taken from each plate and the radioactivity was measured by scintillation counter (LS 6000SE, Beckman). This was designated as the residual radioactivity at "time zero", symbolized as T_0c for control plates and T_0p for ponasterone A-treated plates. The cells were incubated for further 24 hr or 48 hr. At each time point, 1 ml of medium was taken from each plate and the radioactivity was measured and designated as the radioactivity at each time point, symbolized as $T_{24}c$, $T_{24}p$, $T_{48}c$ and $T_{48}p$, respectively. Total protein degradation (designated as D) for each plate was determined as follows: $D_{24}c = T_{24}c - T_0c$,

D_{24p}= T_{24p}- T_{0p}, $D_{48}c$= $T_{48}c$- T_{0c} and D_{48p}= T_{48p} - $T_{0}p$. The total protein degradation rate (designated as R) caused by the over-expression of our gene-of-interest was calculated as: R_{24}=(D_{24c}- D_{24p})/ D_{24c}*100%, R_{48}=($D_{48}c$- D_{48p})/ $D_{48}c$*100%.

Protein Extraction from Cell Culture

M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) was used to extract protein from the myotubes. The manufacturer's protocol was followed with some modifications. Briefly, culture medium was removed and 5ml PBS (0.1 M phosphate and 0.15 M NaCl, pH7.2) were used to wash the cells twice. Then all plates were put on ice until all lysate was collected. A solution of pre-dissolved protease inhibitor cocktail tablet (Roche Diagnostics GmbH, Mannheim, Germany) was added to M-PER reagent. M-PER reagent (700 ul) was added to each plate and each plate was gently rocked for 5 min. A rubber policeman was used to help the collection of all lysate. We then transferred the lysate to a microcentrifuge tube and centrifuged the samples at 13,000 rpm for 10 min at 4°C. An aliquot of the supernatant was placed into small tubes and stored at -80°C. Protein concentration was measured by the Bio-Rad Protein Assay method (Bio-Rad, Hercules, CA).

SDS-PAGE and Immunoblotting

A total of 20 ug protein extract were loaded in each lane on SDS-PAGE gel (100V, 2 hr) in a PROTEAN II Mini-Gel Vertical Electrophoresis unit (Bio-Rad) with a Tris-glycine running buffer system (25 mM Tris-base, 129 mM glycine, 0.1% (w/v) SDS (pH 8.3)). Kaleidoscope (Bio-Rad) pre-stained protein standard was used. A Trans-Blot Cell unit (Bio-Rad) and a Tris-glycine buffer system with addition of 20% methanol without SDS were used for membrane transfer. The proteins were transferred onto an OPTITRAN membrane (nitrocellulose, Schleicher & Schuell, Keene, NH) at 20 V for 20 min and 100 V for 1 hr. A 4% stacking gel and various percent separating gels were used for

separating different proteins. A 5% separating gel was used for spectrin; while a 7.5% separating gel was used for m-calpain, u-calpain, 30k small subunit, desmin and tropomyosin.

Typical Western blotting protocol (103) was followed with some modifications. Briefly, we removed the membrane blot and blocked the non-specific sites with a blocking buffer (5% skim milk, TTBS: 50 mM Tris-base, 150 mM NaCl and 1%Tween-20) for one hour at RT with shaking. We then incubated the blot with the primary antibody in TTBS plus 5% skim milk for 1 hr with shaking. The primary antibodies were: mouse anti-spectrin (1:10,000, Chemicon, Temecula, CA), mouse anti-m-CL (1:10,000, BioMol, Plymouth Meeting, PA), mouse anti-u-CL (1:10,000, BioMol), rabbit anti-30K (1:5,000, generously provided by Dr. Elce, Queen's University, Canada), and mouse anti-desmin (1:10,000, Sigma), mouse anti-tropomysin (1:10,000, Sigma). We washed the membrane with TTBS four times (15 min each) following antibody probing and incubated the blot in TTBS plus 5% skim milk with HRP-conjugated secondary antibody (goat-anti-mouse IgG-HRP conjugated, 1:10,000, or goat-anti-rabbit IgG-HRP conjugate, 1:10,000) for 1 hour at RT with shaking. The membrane then was washed with TTBS four times (15 min each). The blot was immersed in Super Signal West Femto Substrate Working Solution (Pierce, Rockford, IL) for 5 min, after which we removed the blot from the substrate solution and placed it in a membrane protector. Bubbles between the blot and the surface of the membrane protector were removed. We then placed the blot against film (Fuji X-ray film, Stamford, CT) and exposed it for an appropriate time (from 5 seconds to 2 min). Blots that have been shown here represent one of at least three similar results. Blots were quantified by the Kodak ID Image Analysis Software (Rochester, NY).

Statistical Analysis

A simple paired t-test was utilized to evaluate difference between individual treatment means (NCSS, Kaysville, Utah). A significant level of 5% was adopted for all comparisons.

Results

Selection of Cell Lines

After tranfection, cells were selected for their resistance to both zeocine and G418. Following this, their differentiation capacity was tested. Only the transfectants which displayed capacity for differentiation were used for further experiments. Reverse-transcription PCR (RT-PCR) was generally used to screen the clones in each cell line to assess the expression efficiency of each gene construct. Only one clone which displayed the highest expression of the gene construct was selected in each cell line for functional assays.

The cell lines which we developed were:

Cell Line Name	Gene of Interest
L8/DN	Dominant negative u-calpain
L8/AnsL	Antisense u-calpain (Large subunit)
L8/AnsS	Antisense 30KD subunit (Small subunit)
L8/AnsLS	Antisense 80K/30K fused construct.

Validation of the L8/DN Cell Line

In our first study, it was necessary to confirm that genes we were attempting to regulate were actually expressed and that these brought about changes in the calpain system. Figure 2.1 depicts the first of these studies where we evaluated the dominant negative (DN) cell line. Here, we assessed effects of adding ponasterone A (inducer of the ecdysone system) to cultures of L8 myotubes on expression of myc-labelled DN-u-calpain (Panel A) and on expression of u-calpain mRNA (Panel B). Confirmation that

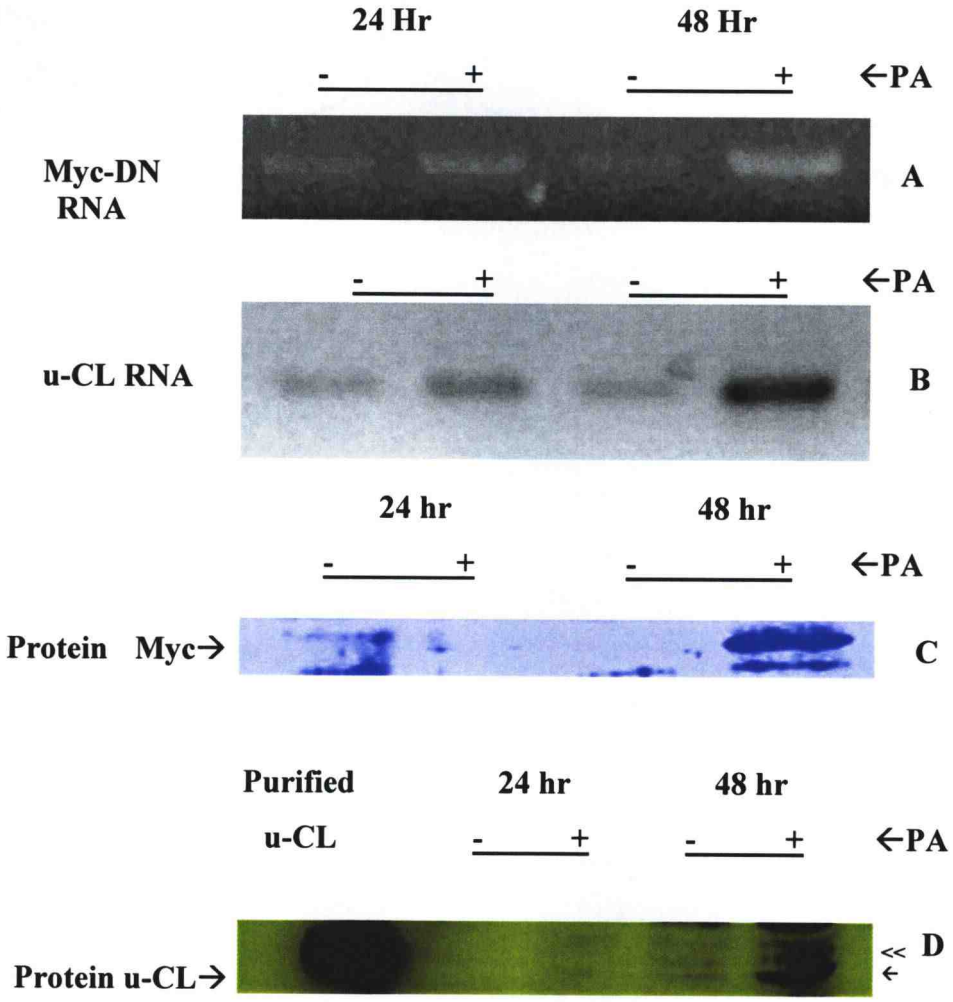

Figure 2.1. Effects of ponasterone A (PA) on Myc-DN mRNA (Panel A) and u-calpain mRNA (Panel B) in L8/DN cells. After adding PA to treated group or equal amount of vehicle to the control group for 24 hr or 48 hr, total RNA was extracted from four groups. Reaction conditions are described in the Materials and Methods section. Western blotting was performed to detect the protein level of Myc-DN-u-calpain (Panel C) and endogenous u-calpain (Panel D, band indicated by arrow). Representative blottings are shown here. Myc-DN-u-calpain was detected as well (Panel D, band indicated by arrow head). The position is exactly the same as Myc when the anti-Myc antibody was used to detect Myc-DN-u-calpain (Panel C).

expression of myc-labelled DN-u-calpain caused expression of myc-u-calpain at the protein level was evaluated by Western blotting (Panel C). Confirmation that over-expression of DN-u-calpain brought about an increase in total u-calpain protein (endogenous + myc-labelled) also was completed (Panel D).

Because there was only one amino acid difference (alanine vs. cysteine) between DN-u-calpain and endogenous u-calpain, there is not an efficient method to distinguish their relative expression. To enable us to detect expression of DN-u-calpain, we expressed a Myc-tagged protein. After Myc-tagging of the DN-u-calpain, it was possible to distinguish the relative expression (at the mRNA level and the protein level) of Myc-tagged DN-u-calpain and u-calpain. A unique primer set was used in RT-PCR: one primer is in the Myc sequence and another is within the u-calpain sequence. Compared with the controls, expression of Myc-DN-u-calpain was detectable when ponasterone A was added to culture media (Figure 2.1, Panel A). After 24 and 48 hr induction, expression of myc-tagged DN-u-calpain was increased by 185% ($P<0.05$) and 230% ($P<0.01$), respectively. The signal in the control lanes (Panel A) represents the "leakage" of the expression system. Leakage in expression was low. The RT-PCR primers in the u-calpain sequence could not discriminate between the DN-u-calpain and the endogenous u-calpain. Hence, both of them were amplified. The RT-PCR product in induced samples is much higher (150% and 170% after 24 and 48 hr induction) than that in controls because of the expression of DN-u-calpain is included (Figure 2.1, Panel B). These data indicated successful expression of myc-tagged DN-u-calpain and u-calpain at the RNA level.

Mouse anti-Myc antibody was used to detect Myc-DN-u-calpain at the protein level. After ponasterone A was added for 48 hr, the Myc-DN-u-calpain protein increased about 250% ($P<0.05$; Figure 2.1, Panel C). Leaky translation in control cells was too low to be detected. In addition to the use of pre-stained protein as a standard, purified u-calpain with 30K subunit was also used as standard. This served as a positive control for monitoring Western blotting. After 48 hr of ponasterone A treatment, total u-calpain protein (a combination of endogenous u-calpain and DN-u-calpain) was increased by

139% and the Myc-DN-u-calapain was increased by 143% (Figure 2.1, Panel D). Again, these data indicated that the L8/DN cell line expressed the gene of interest appropriately at both mRNA and protein levels.

Validation of Antisense Expressing Cell Lines (L8/AnsL, L8/AnsS and L8/AnsLS)

Expression of antisense mRNA can significantly impair the expression of an endogenous gene. Three different forms of antisense mRNA were designed to down-regulate the endogenous calpain subunits. Antisense u-calpain was designed to reduce u-calpain 80KD subunit. Antisense 30KD subunit was designed to reduce the calpain 30KD subunit concentration and, possibly, both u-and m-calpain activities. A fused antisense construct (u-calpain 80KD and 30K) was also designed to reduce both the 30KD and u-calpain subunits. All antisense fragments were expressed at full length. RT-PCR could not distinguish the difference between sense and antisense mRNAs. Compared with the controls, however, the endogenous calpain subunit mRNAs (i.e., 30KD and u-calpain) in induced plates should be lower because the antisense mRNA causes the degradation of sense mRNA. Therefore, we assessed effects of ponasterone A on mRNA encoding 30KD and u-calpain mRNA to verify efficacy of our antisense cell lines.

We evaluated effects of the three antisense constructs on concentrations of 30KD and u-calpain subunits at mRNA and protein levels. Expression of antisense u-calpain significantly reduced concentration of the endogenous u-calpain mRNA by 41% ($P<0.05$) after 24 hr induction and by 26% ($P<0.01$) after 48 hr induction (Figure 2.2, Panel A). Expression of antisense 30KD subunit in L8/AnsS cells effectively reduced 30KD mRNA concentration by 9.4% and 9.6% after 24 and 48 hr indcution (Figure 2.2, Panel B), respectively. Over-expression of the fused antisense construct in L8/AnsLS for 24 hr did not significantly affect u-calpain and 30KD subunit mRNAs. After 48-hr of induction, however, the endogenous u-CL mRNA level was reduced about 60% (Figure 2.2, Panel C) and the 30KD about 45.2% (Figure 2.2, Panel D) by expression of the fused antisense.

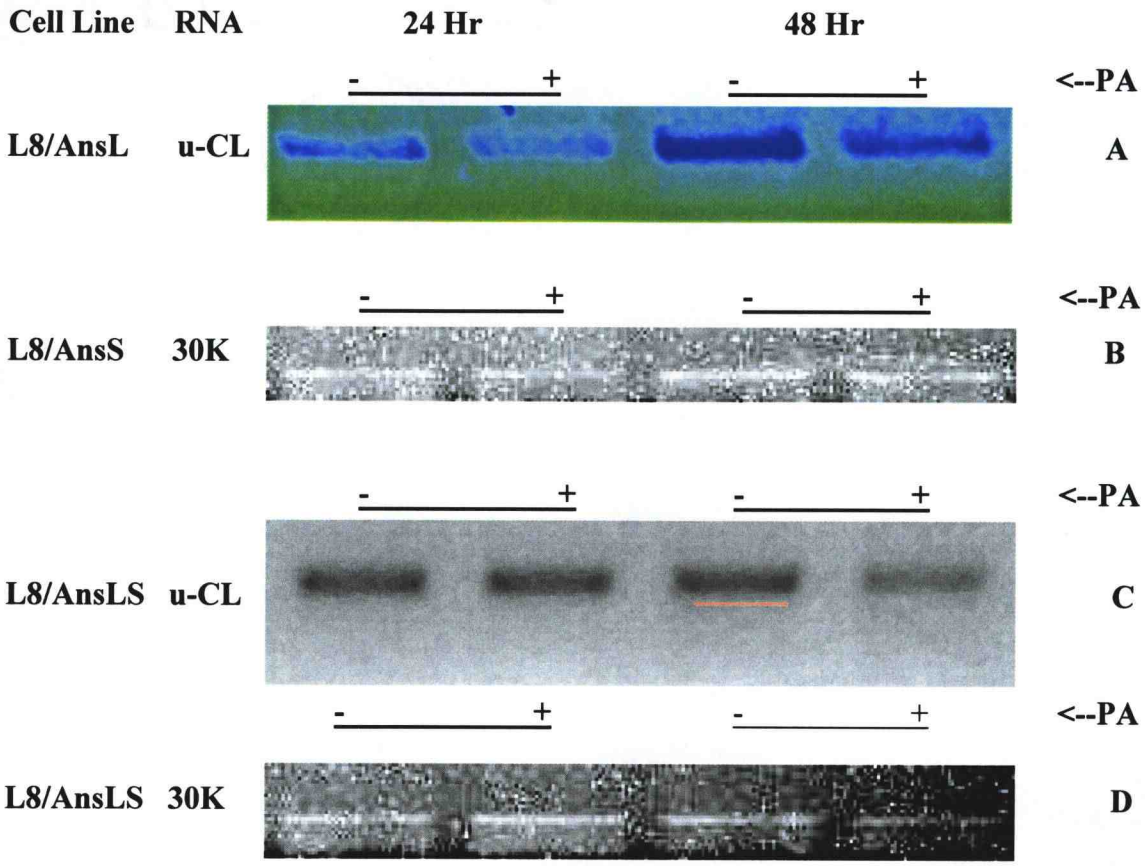

Figure 2.2. Effects of expression of antisense u-calpain and 30KDa on mRNAs encoding these two subunits. The treatments are the same as mentioned in the Figure 2.1. On the left side of each gel, the cell line name and the RT-PCR product name are indicated. On the right side of each gel, panels A-D are indicated. Ponasterone A was added for 24 or 48 hr to lanes marked with "+". Other treatments were exposed to vehicle only.

A change in mRNA level doesn't necessary mean the same or even similar changes will occur at the protein level. In order to address this question, Western blotting was performed. Mouse anti-u-calpain monoclonal antibody was used to assess u-calpain protein, and rabbit anti-30K serum (a gift from Dr. John Elce, Queen's University, Canada) was used to assess concentration of the 30KD calpain. Purified u-calpain (Gibco) was used as a positive control. Clearly, expression of antisense u-calpain mRNA significantly reduced endogenous u-calpain concentration at 24 hr (34.4%) and 48 hr (33.9%) time-frames (Figure 2.3, Panel A). Similarly, expression of antisense mRNA against the 30K calpain subunit reduced 30K protein level by 10.3% and 2.4% at 24 and 48 hr time-frames (Figure 2.3, Panel B), respectively. Over-expression of 80K/30K antisense did not reduce u-calpain (Figure 2.4, Panel A) and 30KD (Figure 2.4, Panel B) at the protein level. This effect was inconsistent with its effect at the mRNA level.

Does Expression of One Calpain Subunit Affect Expression of Related Subunits?

Most research on calpains has focused on the two house-keeping large subunits (u- and m-calpain 80KDa). Relatively few studies have involved the 30KD subunit, as done in this study. A goal of our research was to study the interrelationship of all calpain subunits and to assemble an understanding of calpain regulation. To do this, our preliminary cell validation studies proved we had the ability to regulate individual calpain subunits and thereby study their relationships to each other. In the first experiment we tested whether alterations in u-calpain expression (by over-expressing Myc-DN-u-calpain) brought about changes in 30KD calpain subunit concentration. Over-expressing Myc-DN-u-calpain increased 30KD calpain subunit concentration at both the mRNA (Figure 2.5, Panel A) and protein (Figure 2.6, Panel A) levels. Specifically, after 24 and 48 hr induction, u-calpain mRNA was increased by 81% ($P<0.07$) and 27.4% ($P<0.01$), and u-calpain protein by 116.7% ($P<0.01$) and 59.3%, respectively. When we reduced u-calpain expression in cells by over-expressing u-calpain antisense, 30KD calpain subunit concentration was reduced at mRNA level (Figure 2.5, Panel B), but was not consistent at protein level (Figure 2.6, Panel B) levels. After 24 and 48hr induction, 30KD mRNA

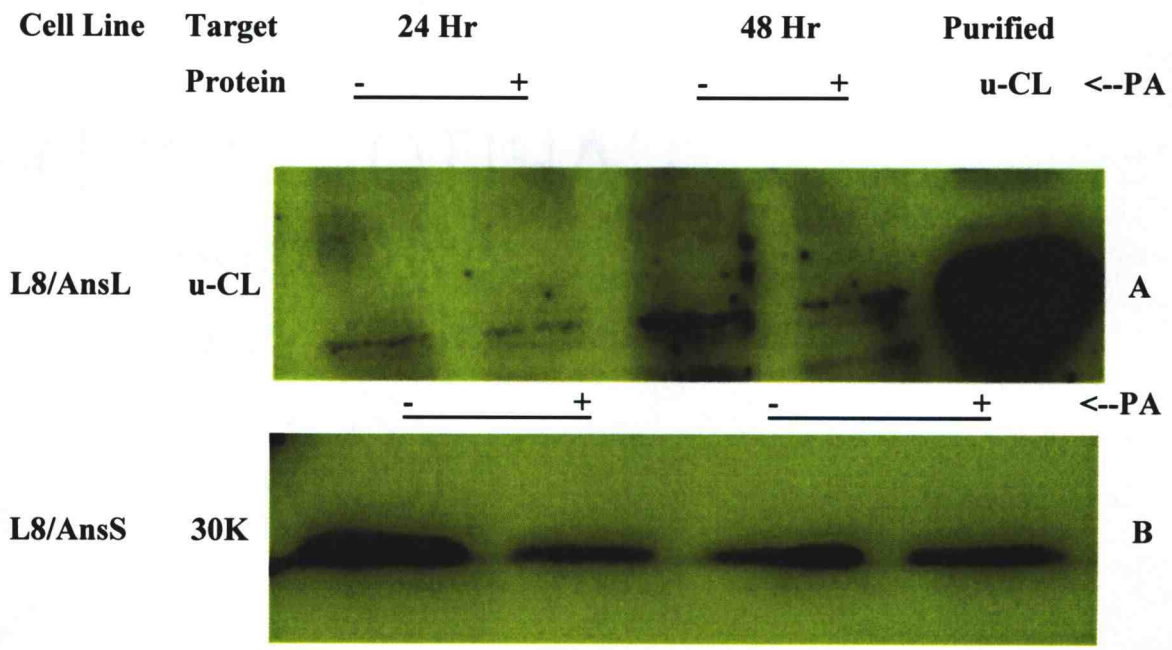

Figure 2.3. Effects of over-expressing antisense u-calpain and 30K on the endogenous u-calpain and 30K at the protein level. On left side of the blot, the cell line name and the target protein for this immunoblotting are indicated. Purified u-calpain was used as positive control (Panel A). Ponasterone A was added for 24 or 48 hr to lanes marked with "+". Other treatments were exposed to vehicle only.

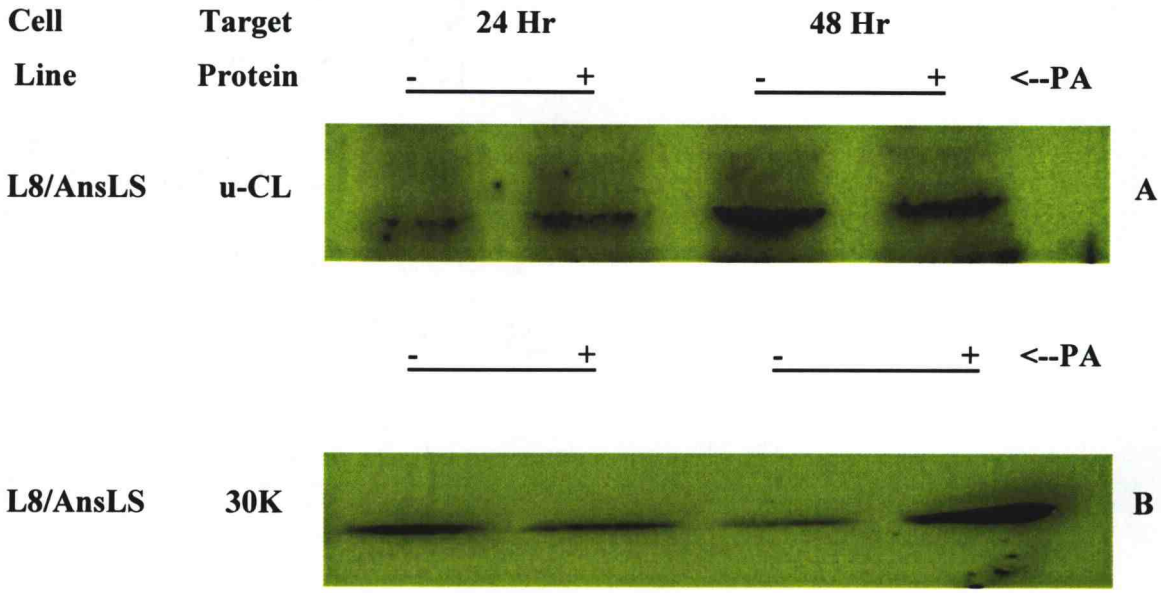

Figure 2.4. Effects of expressing fused 80K/30K antisense on u-calpain and 30KDa subunit protein concentration. On the left side of the blot, the cell line names and target protein of the Western blotting are mentioned. Ponasterone A was added for 24 or 48 hr to lanes marked with "+". Other treatments were exposed to vehicle only.

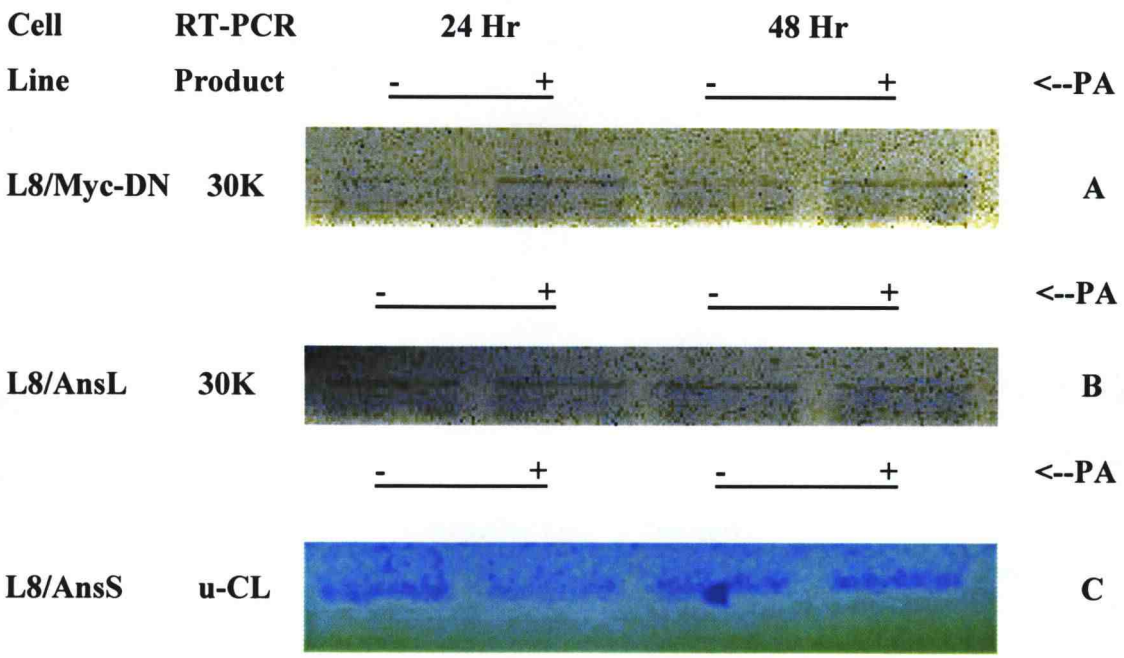

Figure 2.5. Effects of expressing DN-u-calpain, antisense u-calpain and antisense 30KDa subunit on 30KD calpain mRNA concentrations. RT-PCR was utilized to measure mRNA concentration. On the left side of each gel, the cell line name and the target gene are mentioned. Ponasterone A was added for 24 or 48 hr to lanes marked with "+". Other treatments were exposed to vehicle only.

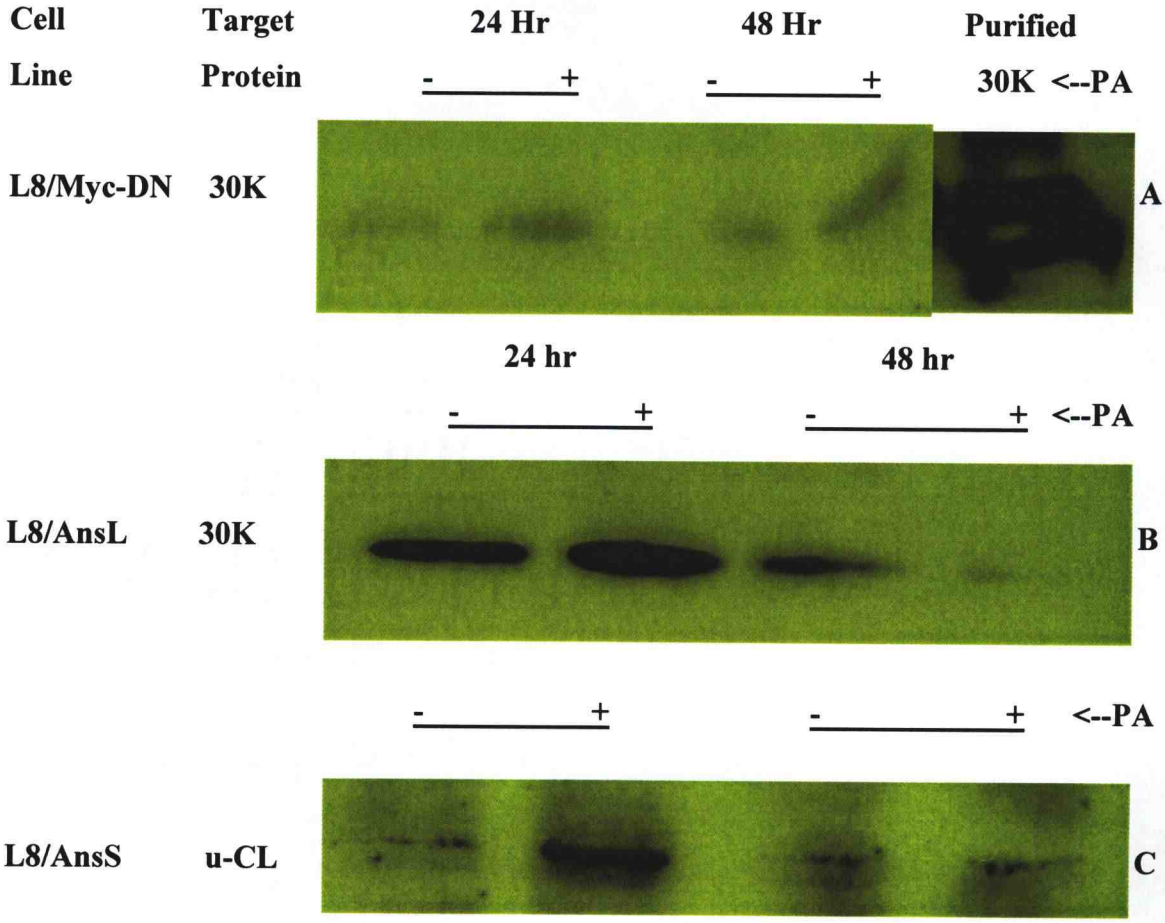

Figure 2.6. Effects of expressing DN-u-calpain, antisense u-calpain and antisense 30K calpain subunit on 30KDa calpain and u-calpain subunit at the protein level. Western blotting was used to assess protein level. Ponasterone A was added for 24 or 48 hr to lanes marked with "+". Other treatments were exposed to vehicle only.

was reduced by 16% and 4%, however, 30KD protein was increased by 11% after 24 induction and was reduced by 93% ($P<0.01$) after 48 hr induction. The data indicated that when u-calpain mRNA is elevated (by over-expressing DN-u-calpain), 30KD mRNA is coordinately increased (or vice versa). This would ensure linkage of the expression of the two components of the calpain heterodimer. Similarly, when we reduced 30KD calpain expression using antisense 30KD expression, even though u-calpain was not affected at mRNA (Figure 2.5, Panel C), u-calpain protein was reduced by 71.9% ($P<0.05$) after 48 hr induction (Figure 2.6, Panel C).

We also investigated relationships between the 30KD and u-calpain subunit with the high-Ca^{2+}-requiring subunit: m-calpain (m-CL). To accomplish this, we evaluated effects of our antisense and DN-u-calpain cell lines on m-calpain expression. We found that expression of the fused antisense (u-CL-30KD) and DN-u-calpain did not affect m-calpain expression at mRNA or protein levels (Figure 2.7, Panels B, D and Figure 2.8, Panels B, D). However, expression of antisense 30K calpain subunit did not affect m-calpain mRNA level (Figure 2.7, Panel C), m-calpain protein level was significantly affected (Figure 2.8, Panel C). Namely, after 24 and 48 hr induction, m-calpain protein increased by 310% ($P<0.01$) and 280% ($P<0.01$), respectively. Over-expression of antisense u-calpain did not affect m-calpain mRNA (Figure 2.7, Panel A), but m-calpain was reduced at the protein level by 55% ($P<0.01$) following 48 hr of induction (Figure 2.8, Panel A). However, expression of fused 80K/30K antisense did not affect the expression of m-calpain (Figure 2.7 and 2.8, Panel D). These data indicate that m-calpain subunit expression, like u-calpain and the 30KD calpain subunit, is coordinately regulated with other calpain subunits. It is not clear whether these changes are mediated directly or indirectly or via changes in mRNA or protein concentration.

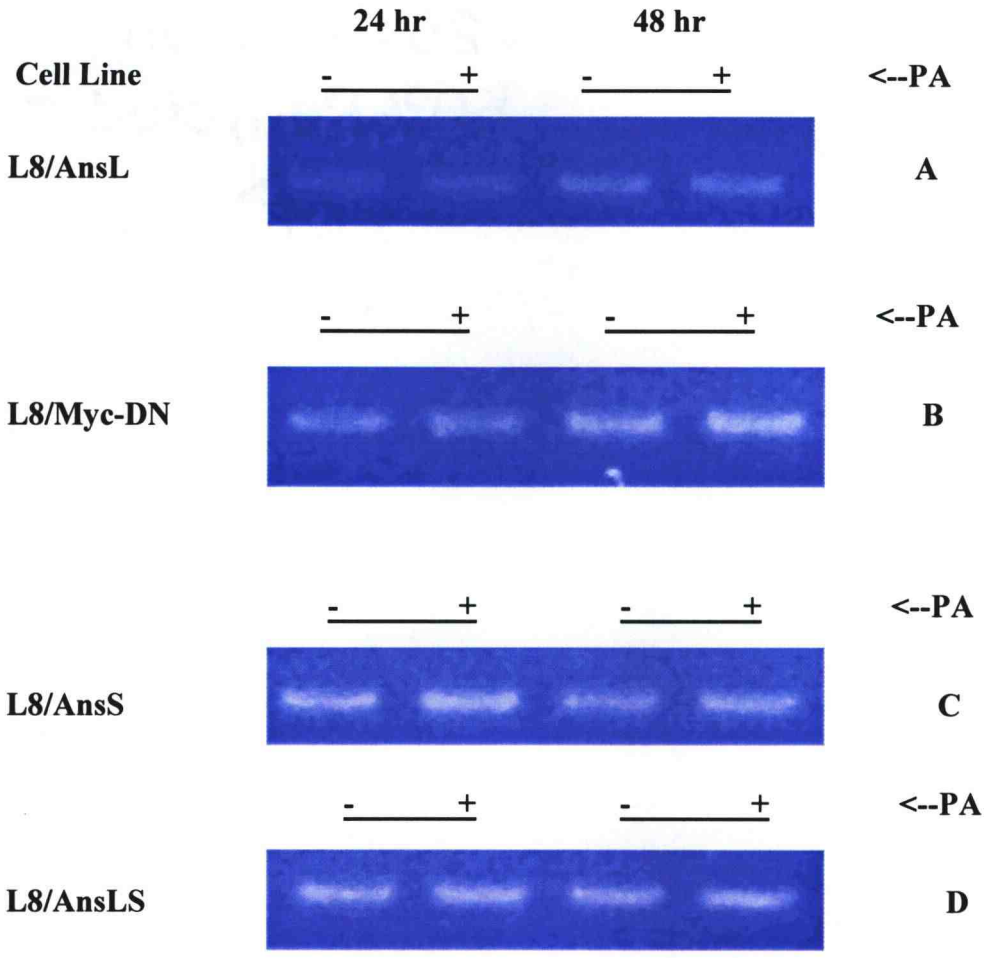

Figure 2.7. Effects of over-expressing antisense u-calpain, DN-u-calpain, antisense 30KDa subunit and the fused u-calpain-30KDa fragment on m-calpain mRNA. 100ng of total RNA was used in 50ul RT-PCR reaction. 10ul of the final product was loaded in each lane. On the left side of each gel, the cell line name is indicated. Ponasterone A was added for 24 or 48 hr to lanes marked with "+". Other treatments were exposed to vehicle only.

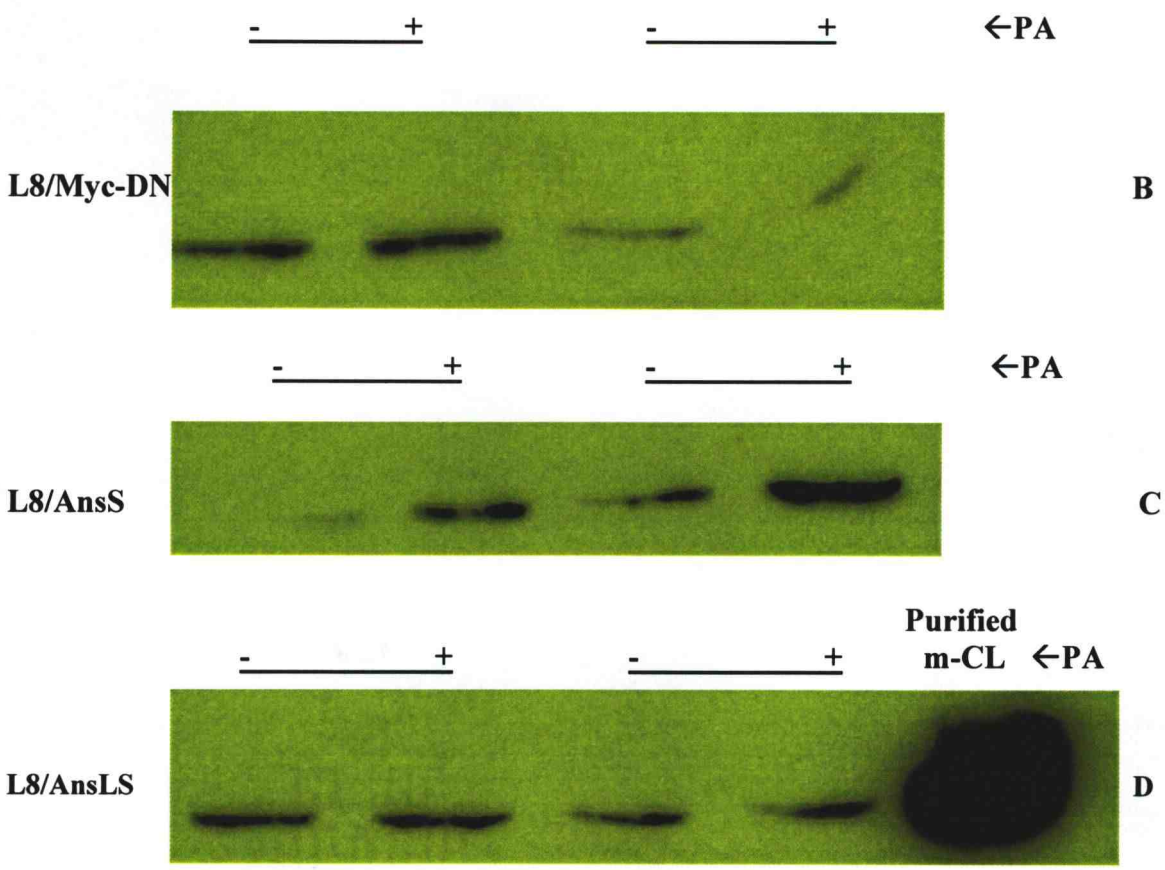

Figure 2.8. Effects of over-expressing antisense 80KD u-calpain (Panel A), DN-u-calpain (Panel B), antisense 30KDa subunit (Panel C) and the fused antisense u-calpain-30K (Panel D) on m-calpain protein level. Mouse anti-m-calpain monoclonal antibody was used and purified m-CL was used as positive control in Panel D. Cell line names are marked on the left side of blots. Ponasterone A was added for 24 or 48 hr to lanes marked with "+". Other treatments were exposed to vehicle only.

Roles of Calpains in Muscle Cells

In addition to establishing the interrelationships between the various calpain subunits, our cell lines permitted us to investigate potential calpain substrates (i.e., calpain function). By reducing activities of calpains in living cells, we can ascertain roles of calpains *in vivo.*

The first substrate we studied was fodrin. Fodrin (spectrin) is a well-established calpain substrate (61, 62, 63). Hence, change in fodrin cleavage in our individual cell lines provides evidence of our ability to regulate calpain activity in muscle cells. In addition, we also studied roles of calpain in total protein degradation and in degradation of tropomyosin and desmin.

Fodrin Degradation by u-Calpain in Rat Myotubes.

Fodrin is a well-established substrate for calpains (61, 62, 63). Accumulation of fodrin breakdown product (BDP, 145/150 KDa) and fodrin concentration itself have been used as indexes for the activity of calpains. In order to evaluate the efficiencies of our cell lines, immunoblotting was performed by using mouse anti-fodrin monoclonal antibodies following ponasterone A induction of the individual cell lines. After 24 and 48 hr induction, fodrin breakdown products were reduced in ponasterone A-treated cells compared to the controls (Figure 2.9, Panels A through D). And, fodrin itself accumulated in some samples. We can conclude that all of our constructs work efficiently in down-regulating the activity of endogenous calpain.

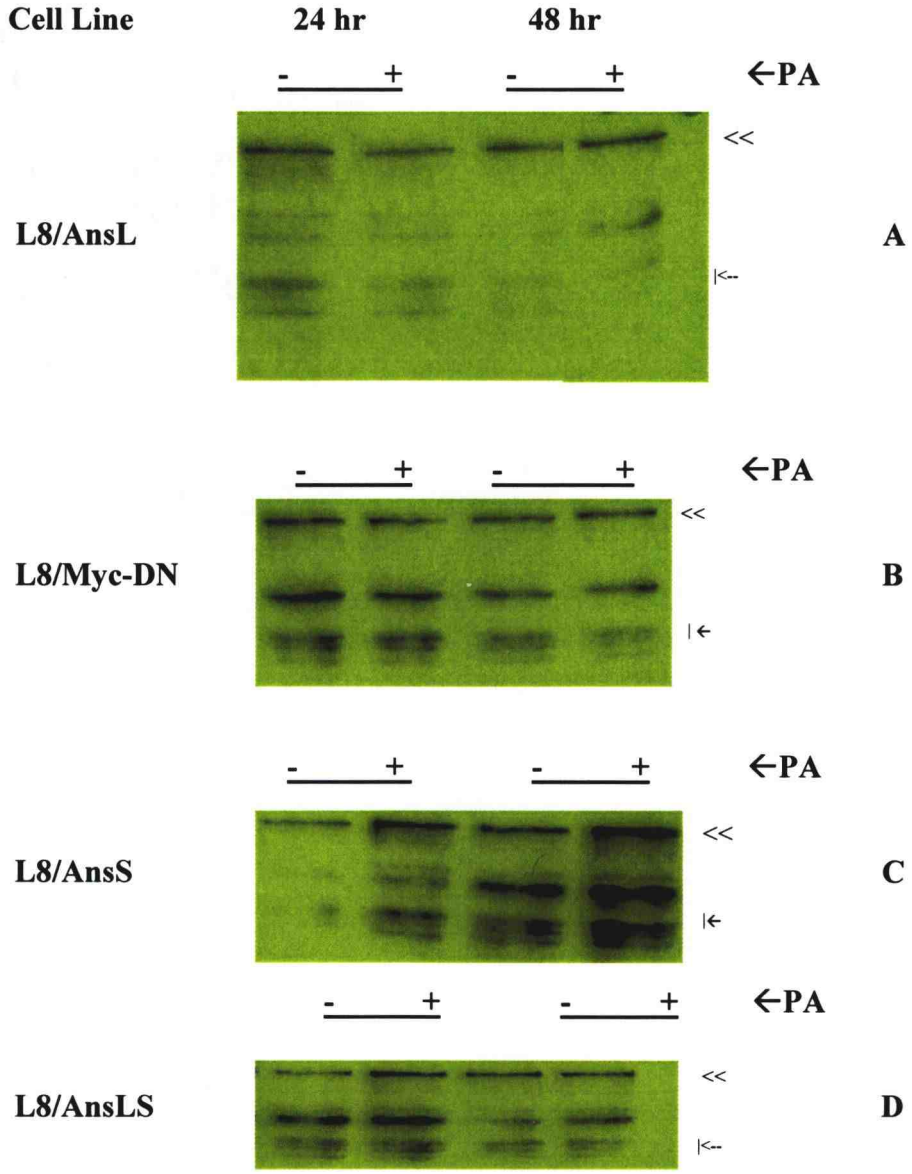

Figure 2.9. Effects of over-expressing antisense u-calpain (Panel A), DN-u-calpain (Panel B), antisense 30KDa subunit (Panel C) and fused antisense u-calpain-30KDa (Panel D) on fodrin degradation. Cell line names were marked on the left side of each blot. The double arrow head (<<) points to the fodrin band. The bar and arrow (|←) points to the 145/150KDa fodrin degradation products. Ponasterone A was added for 24 or 48 hr to lanes marked with "+". Other treatments were exposed to vehicle only.

Effects of Calpain Activity on Total Protein Degradation in Rat Myotubes

After proving ability to down-regulate calpain activity, we evaluated the role of u-calpain in skeletal muscle total protein degradation. Our results showed that u-calpain plays a significant role in skeletal muscle total protein degradation. Specifically, over-expressing u-calpain antisense mRNA reduced total protein degradation by 6.4% ($P<0.05$) following 24 hr of ponasterone A treatment and by 11.9% ($P>0.05$) following 48 hr of ponasterone A treatment. Over-expression of DN-u-CL reduced total protein degradation by 7.9% ($P<0.01$) following 24 hr of ponasterone A treatment and by 10.6% ($P<0.01$) following 48 hr of ponasterone A treatment. Over-expression of antisense 30KD subunit reduced total muscle protein degradation by 13.4% ($P<0.05$) following 24 hr of ponasterone A treatment and by 7.3% ($P>0.05$) following 48 hr of ponasterone A treatment. Over-expression of the fused antisense construct reduced total muscle protein degradation by 3.5% and 3% at 24 hr and 48 hr time points ($P>0.05$), respectively.

Role of Calpain in Desmin Degradation in Rat Myotubes

Desmin is an intermediate filament which can be degraded by calpains rapidly *in vitro* (71). In order to determine whether desmin is a true substrate of calpains, we evaluated effects of over-expressing antisense u-calpain (Figure 2.10, Panel A), Myc-tagged-DN-u-calpain (Panel, B), antisense 30KD subunit (Panel C) and the fused antisense 80K/30K calpain fragment on desmin concentration following 24 and 48 hr of ponasterone A administration. Clearly, all cell lines demonstrated increased desmin concentration (stabilization) when calpain activity was reduced. Specifically, after 48 hr induction, desmin protein concentration was increased by 170.8% in L8/AnsL cell line, 30% in L8/DN cell line, 138.7% ($P<0.01$) in L8/AnsS cell line, and 564% ($P<0.01$) in L8/AnsLS cell line, respectively. These data support the contention that desmin is a true calpain substrate.

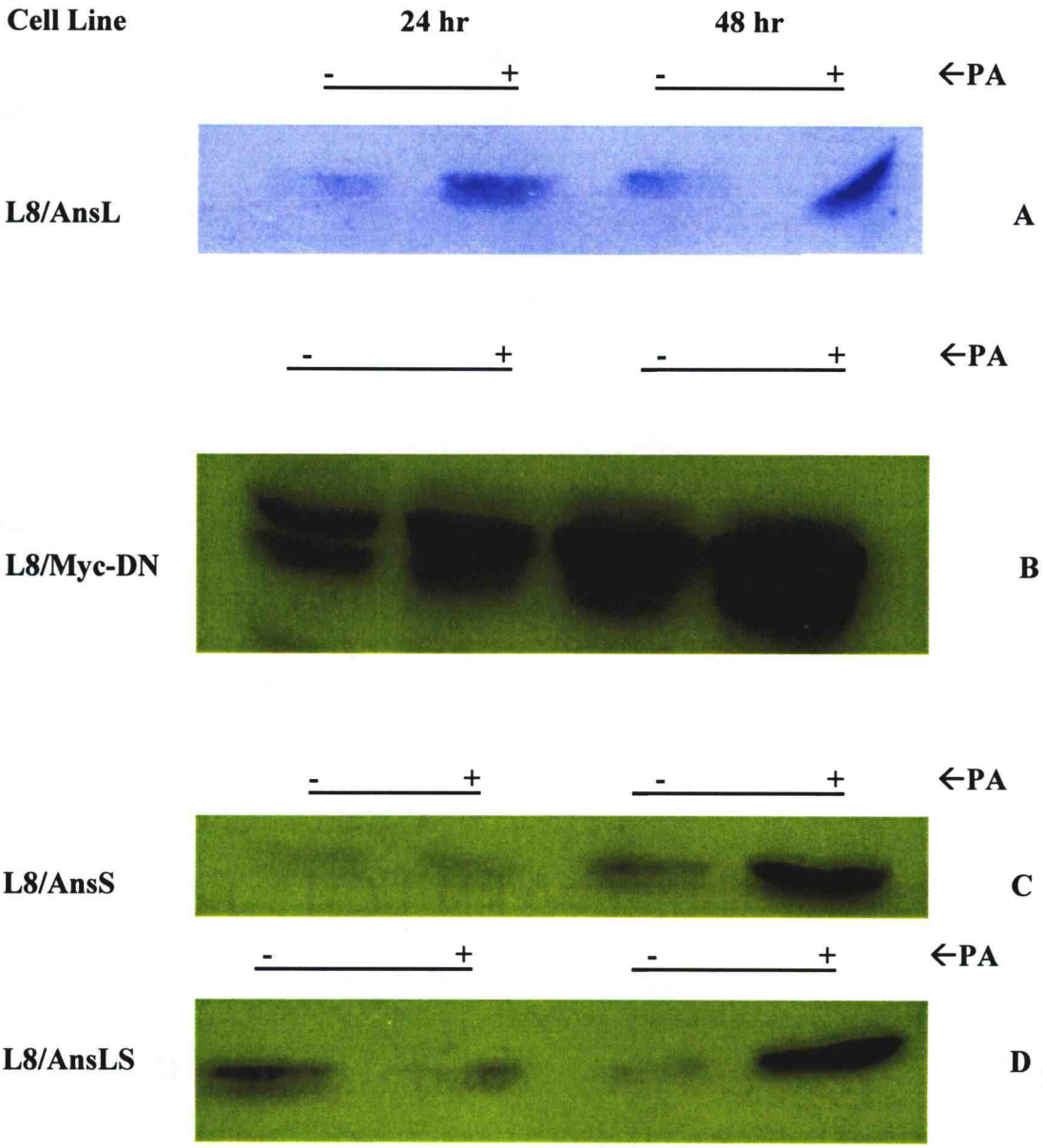

Figure 2.10. Effects of over-expressing antisense u-calpain (Panel A), DN-u-calpain (Panel B), antisense 30KDa subunit (Panel C) and fused antisense u-calpain-30KDa (Panel D) on desmin concentration. The cell line name is marked on the left side of each blot. Mouse anti-desmin monoclonal antibodies was used to detect desmin protein. Ponasterone A was added for 24 or 48 hr to lanes marked with "+". Other treatments were exposed to vehicle only.

Role of Calpain in Tropomyosin Degradation in Rat Myotubes

In a strategy identical to that used for desmin, we evaluated the role that calpain has in degradation of tropomyosin (Figure 2.11, Panels A to D). Others (72) have indicated, based on *in vitro* studies, that tropomyosin is a calpain substrate. However, we found that tropomyosin concentration was unaffected by ponasterone A treatment in any of our cell lines. These data indicate that tryopomyosin is not a calpain substrate.

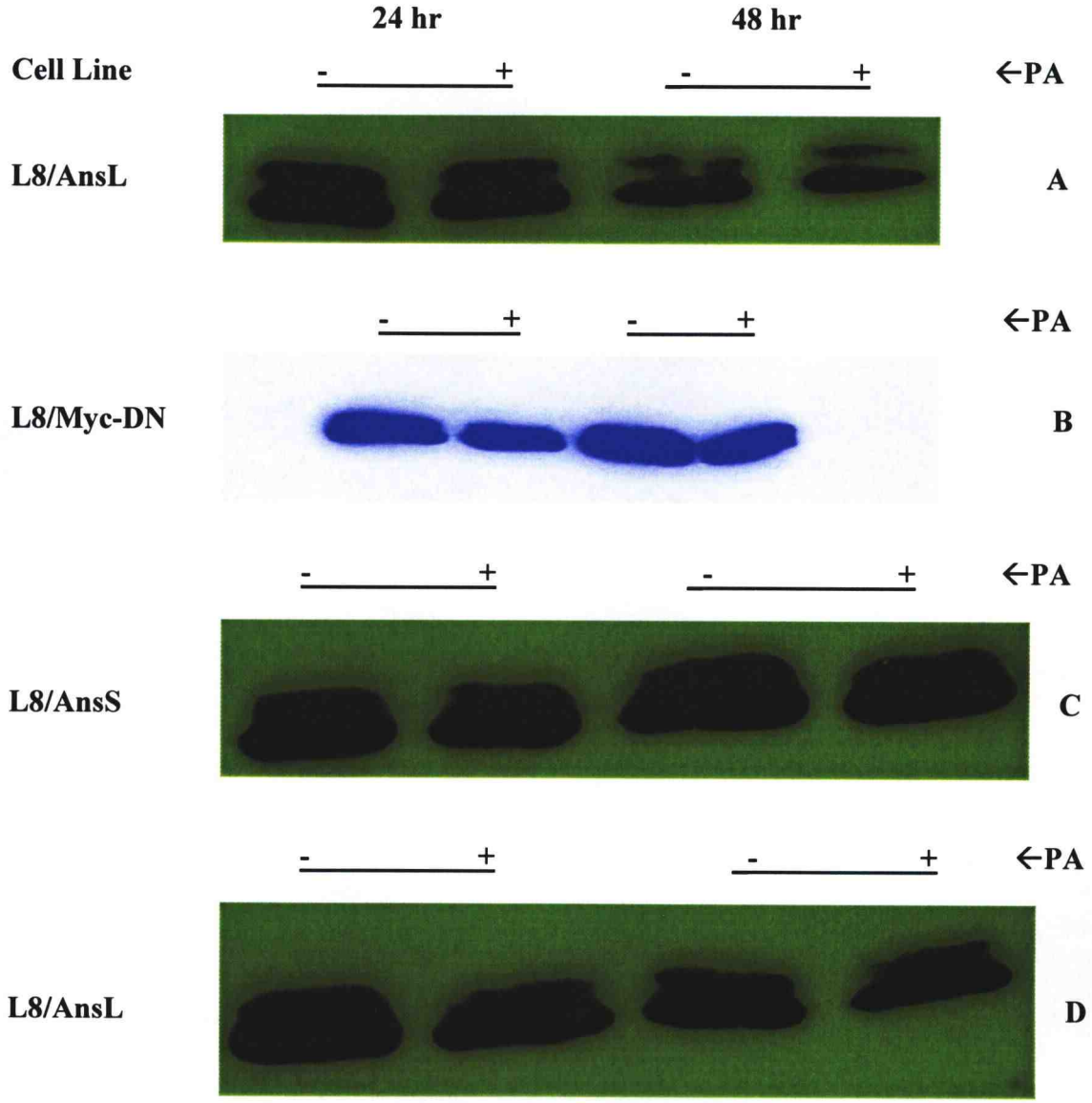

Figure 2.11. Effects of over-expressing antisense u-calpain (Panel A), DN-u-calpain (Panel B), antisense 30KDa subunit (Panel C) and fused antisense u-calpain-30KDa (Panel D) on tropomyosin concentration. Cell line names are marked on the left side of each blot. Ponasterone A was added for 24 or 48 hr to lanes marked with "+". Other treatments were exposed to vehicle only.

Discussion

Validation of the Cell Lines

In the first phase of this study, we systematically examined relationships between the most prevalent forms of the calpain subunits (80KD u- and m-calpain subunits and the 30kD small subunit) in skeletal muscle cells. Inter-relationships were assessed at the transcriptional (mRNA concentration) and translational (protein concentration) levels. The study was completed without application of any stimuli that might stimulate or inhibit protein degradation. Hence, the results should reflect the dynamics among the subunits in normal, non-stimulated muscle cells.

To accomplish this, we developed a variety of cells lines in which the various components of the calpain system could be regulated via the ecdysone expression system. This constituted the first effort to apply the ecdysone expression system to muscle cells. Here, ponasterone A was added as an inducer to culture media. To ascertain the validity of these approaches, we assessed abilities of the transgenic cell lines to regulate calpain subunit expression and also assessed fodrin degradation. Fodrin is a well-known calpain substrate and its protection is interpreted as evidence of a reduction in calpain activity (61, 62, 63). It's perhaps the best measure, albeit indirect, of calpain activity in living cells

In this first study, we documented that the DN-u-calpain cell line expressed DN-u-calpain, as indicated by the increase of specific Myc-DN-u-calpain signal, when ponasterone A was added to media (Figure 2.1, Panels A and C and D) and that this resulted in reduction in endogenous u-calpain activity, as demonstrated by the stabilization of fodrin (Figure 2.9, Panel B). These data demonstrate that the DN-u-

calpain cell line expresses a protein (a "dead" calpain) which reduces endogenous u-calpain activity. And, we propose that we may use this line to evaluate the roles of u-calpain in muscle cells. In other studies (19) we successfully applied a similar approach (expression of a DN-m-calpain) to elucidate roles of m-calpain in muscle cells (using a LacSwitch expression system). We propose that the DN-u-calpain exerts its activity by competing with endogenous active u-calpain for substrate and thereby sequestering substrate from degradation by active endogenous protease.

After 48 hr induction, Myc-DN-u-calpain expression was much higher than endogenous u-calpain (Figure 2.1, panel D). In the construction of Myc-DN-u-calpain, the start codon for DN-u-calpain and the Kozak sequence for u-calpain were not mutated (see "methods and materials" section). Therefore, the translation for Myc-DN-u-calpain mRNA could initiate from the start codon in front of Myc and the translated protein was Myc-DN-u-calpain. Alternatively, the translation of Myc-DN mRNA could start from the original start codon of DN-u-calpain and the translated protein would be DN-u-calpain. Despite this, the DN-u-calpain and endogenous u-calpain will have a molecular size which is very similar and which cannot be resolved on a gel. As a result, an RT-PCR product generated from u-calpain primers represents contribution of both DN-u-calpain and u-calpain mRNAs. Accumulation of mRNA is likely to result from the synthesis of the DN-u-calpain protein.

In a second study, we evaluated effects of over-expressing antisense u-calpain mRNA on u-calpain mRNA and protein levels. As anticipated, addition of ponasterone A to cultures of this cell line increased total u-calpain mRNA (a mixture of both native and antisense u-calpain mRNA, Figure 2.2, Panel A) and brought about a reduction in u-calpain protein (Figure 2.3, Panel A). Also, over-expressing the u-calpain antisense, like DN-u-calpain, stabilized fodrin, a calpain substrate. Hence, this cell line enables us, via a different strategy to DN, to elucidate the roles of u-calpain in skeletal muscle cells.

The 30kD subunit is needed by both u- and m-calpains as the second component of the calpain heterodimer. It is an essential cellular protein because 30KD knock-out mice

demonstrated embryonic lethality(s). However, knock-out 30 KD subunit is not lethal in cell culture (5). Because of its importance to both u- and m-calpain, we propose that reduction of 30 KD subunit would reduce activities of both u- and m-calpains. When we added ponasterone A to cultures of L8/AnsS cells, 30 KD mRNA was significantly reduced (Figure 2.2 Panel B) as was 30 KD subunit at the protein level (24 hours, Figure 2.3, Panel B). Finally, over-expression of the antisense 30 KD subunit stabilized fodrin (Figure 2.9), a well-known calpain substrate. This demonstrates a functional consequence to the regulation of 30 KD calpain subunit expression in skeletal muscle cells.

The final strategy we used to regulate calpain activity in muscle cells was to over-express a fused antisense fragment that consisted of the 80 KD u-calpain and 30 KD subunit. This was a new idea that was proposed by the authors as a means of simultaneously regulatory both 30KD and u-calpain subunits. Over-expressing the antisense 80K/30K fragment reduced u-calpain and 30 KD subunit mRNAs (Figure 2.2, Panels C and D) but did not consistently affect the concentrations of these proteins (Figure 2.4, Panels A and B). Despite this unexpected result, over-expressing the fused antisense fragment stabilized fodrin (Figure 2.9) as did the over-expression of the other fragments. Based on this, it appears that the fused antisense reduces calpain activity. However, the mechanism by which it brings about this change was not clear.

Interactions Between the Calpain Subunits

An interesting feature of the calpains is that there appears to be coordinated expression of their subunits. This makes good sense because the u- and m-calpain subunits require the presence of the 30 KD subunit for heterodimer formation. And, some scientists have proposed that the calpains work as a cascade (45, 46) thus necessitating their coordinated expression. In addition, coordinated expression of the calpains has been indicated in previous studies in our lab where Ilian and Forsberg (87) determined that fasting caused coordinated changes (increases) in all calpain subunits (u-, m- and 30 KD subunits and mRNAs of a variety of other proteases) during fasting in rabbit skeletal muscle. In

another study (88), phorbel ester increased both m-calpain and 30 KD subunit mRNA in HeLa cells. Hypoxia in pulmonary endothelial cells (89) and hippocampal neurons (90) brings about changes in both u- and m-calpains. To date, it is not clear whether calpains bring about changes in their own expression or, alternatively, whether their expression is coordinated by similar transcriptional/translational control mechanisms. Availability of our cell lines enabled us to evaluate the first possibility: that calpains influence expression of one another.

Our results indicate that the calpain subunits affect expression of each other. When we expressed DN-u-calpain, the concentration of the corresponding 30 KD subunit was increased (Figure 2.5, Panel A; Figure 2.6, Panel A). Conversely, when we expressed antisense u-calpain, the concentration of the corresponding 30 KD subunit was also reduced (Figure 2.5, Panel B and Figure 2.6, Panel B). The opposite relationship, however, was revealed when we over-expressed an antisense 30 KD subunit. While we expected reduced 30 kDa subunit concentration would reduce u- and m-calpain activities, expression of both u- and m-calpain was increased at both the mRNA and protein levels (Figure 2.5, Panel C; Figure 2.6 Panel C; and Figure 2.7, Panel C; Figure 2.8, Panel C). Unfortunately, we do not have a simple model that accommodates all of these observations.

Finally, our research revealed a relationship between the expression of u- and m-calpains. Down-regulation of u-calpain with u-calpain antisense expression also reduced m-calpain concentration at both mRNA and protein levels following 48 hr of ponasterone A administration (Figure 2.7, Panel A and Figure 2.8, Panel A). These data are consistent with the view that calpains work together to degrade proteins and that their expressions are coordinated mutually. The next step in this work is to identify the means by which calpains alter expression of the subunits.

Some of our results are opposite of those reported in 30 KD knock-out mice. Specifically, a 30 kD knock-out reduced u- and m-calpain at the protein level (5); however, in our cell lines, down-regulation of the 30 kDa subunit increased both u- and

m-calpain concentrations. We cannot explain these different results except to suggest that the experimental system was quite different and cell lineage studied was different. But one possible explanation is that muscle is different from other tissues. For example, we found that protease mRNAs in muscle were all up-regulated in fasting (87) when they were all reduced in other tissues. Muscle regulation of proteases was opposite all other tissues which we examined (liver, lung and kidney). We proposed this may serve as a basis by which muscle may nourish the entire organism during fasting. It's possible the different response in muscle in this study reflects this possibility.

It is believed that the lethality of the 30 KD knock-out was related to its abolition of calpain activities. One aspect of interest here is that we might rescue 30 kD knock-out embryos using the methods outlined in this thesis (i.e., ponasterone A-inducible expression of 30 KD subunit) and thereby study individual functions of the 30 kD subunit in living animals (91).

Calpain Substrates: What Is Calpain's Role in Total Protein Degradation?

To ascertain the role that u-calpain plays in total protein degradation, we assessed effects of ponasterone A addition to culture media on the rate of release of tritium-labeled tyrosine from myotubular cultures. This study was completed with all of our cell lines. To prevent recycling of released labeled tritium, unlabeled tyrosine was included in a chase medium to enable complete recovery of released radioactivity from the cultures.

Induction of antisense u-calpain expression for 24 hours (but not 48 hours) caused a significant ($P<0.05$) reduction in total protein degradation. The difference was small (6.4%) indicating that u-calpain contributes to only a small portion of total protein degradation in muscle cells. This may be viewed as consistent with u-calpain's reputed role in limited proteolysis, not in complete digestion of proteins to amino acids.

Similar to these results, over-expression of DN-u-calpain, a second strategy to reduce u-calpain activity, reduced total protein degradation by 7.9% ($P<0.05$) following 24 hours of ponasterone A administration and by 10.6% ($P<0.0022$) following 48 hours of ponasterone A administration. These data support a role for u-calpain in total protein degradation and are consistent with the effect of antisense u-calpain expression (preceding paragraph).

We also investigated the effects of over-expression the 30 KDa subunit antisense and the fused 80K:30K antisense fragment on total protein degradation following 24 and 48 hours of ponasteron A administration. Results for the 30 KD antisense expression were consistent with antisense u-calpain and DN-u-calpain. Specifically, over-expressing the antisense 30 KD subunit reduced total protein degradation by 13.4% ($P<0.05$) and 7.3% ($P>0.05$) at 24 and 48 hours, respectively. Over-expression of the fused antisense construct did not significantly ($P>0.05$) alter total protein degradation. These data generally support a proposal that u-calpain contributes to a small, but consistent, aspect of total protein degradation in muscle cells. We are not certain why the fused construct did not affect total protein degradation but point out that its effects were, throughout the study, less spectacular than effects of the individual calpain fragments.

The relatively small effects that down-regulation of the 30 KD subunit had on total protein degradation are not consistent with some of our earlier studies (19). In these studies, when we reduced total calpain activity by over-expressing calpastatin inhibitory domain, we were able to reduce total protein degradation by approximately 60%. Reducing the 30 KD subunit was expected to exert a similar effect (since it was expected to reduce activities of both calpains). From this analysis, we can only conclude that over-expressing the calpain inhibitor (calpastatin) is a much more effective method of inhibiting calpains than over-expressing of 30 KD subunit antisense. A confounding fact that needs to be considered, however, is that in previous studies we used a LacSwitch expression system (19) and, in these studies, we used an ecdysone-based expression system. We were not able to compare the relative levels of expression of these two expression systems. Hence, it may be unfair to directly compare these two data sets.

Does Calpain Degrade Desmin and Tropomyosin?

In the final studies, we evaluated the potential for u-calpain to degrade desmin and tropomysoin. Desmin is an intermediate filament and has been shown to be degraded by calpains *in vitro* (71). However, degradation of a substrate by calpain in a test tube cannot be taken as unequivocal evidence of its role as a substrate in living cells. To test the possibility that desmin is a true calpain substrate, we evaluated the effects of expressing the various calpain fragments on desmin concentration, an index of desmin stability. Accumulation of desmin was taken as evidence for the stabilization (i.e., reduced degradation) of desmin. From our results (Figure 2.10), it is clear that reducing calpain avtivity, whether by DN expression or antisense expression, stabilized desmin. These data indicate that desmin is a calpain substrate in living cells.

Typically, when one considers muscle diseases and the dystrophies, one is reminded of the lack of proteins which manifest themselves as disease. For example, gradual loss of dystrophin is the basis for the most prevalent cause of muscular dystrophy in humans (104). However, myopathies also result from accumulations of unwanted (or excess) proteins such as desmin (desminopathies; 92). The desmin in desmin-containing inclusions is often mutated (missense mutation or deletion mutation; 93-95). In this study, we demonstrated that desmin is a substrate for calpain. Therefore, calpain may be involved in the desminopathy. The potential mechanism might be that mutated desmin may be less susceptible to calpain's degradation. Or, possibly, calpain activity is reduced in desminopathy. These conditions could allow for the accumulation of mutated/normal desmin and formation of filamentous inclusions.

Finally, we evaluated the hypothesis that tropomyosin is a calpain substrate. Tropomysoin is a component of the thin filament and serves to stiffen this filament and interacts with the troponin complex (96). Over-expression of any of our constructs that

interfered with calpain activity did not stabilize tropomyosin (Figure 2.11 Panels A-D). Because tropomyosin did not accumulate when calpain was inhibited we propose that tropomyosin is not a calpain substrate.

Conclusions

We have developed several strategies for regulating calpain activities in muscle cells and have determined that these methods effectively control calpain activities in living cells. Proof of this includes appropriate changes in calpain subunit mRNA and protein concentrations and appropriate changes in fodrin degradation. Based on these encouraging preliminary studies, we then determined that the calpain subunits can influence expression of one another and that u-calpain plays a minor role in total protein degradation in muscle cells. Finally, desmin (but not tropomysoin) is a substrate for calpain. It is possible that defects in calpain-dependent processing of desmin could lead to desminopathy.

In the past two decades, knowledge of the roles of proteases has accumulated from studies in which inhibitors of calpains were used in cell cultures and also where individual proteases were shown to be able to degrade specific purified proteins in test tubes. These types of studies had two significant drawbacks. First, in many cases, the inhibitors used were found to exert non-specific actions on other proteases. For example, neuroprotective effects of calpain inhibitors have been studied in traumatic and ischemic brain injury (97-99). Calpain inhibitors have also been used to study spectrin breakdown and inhibition of apoptosis (103, 100, 101). With present knowledge, some of the effects of the inhibitors used in these studies may have resulted from inhibition of other proteases such as the proteasome (102). In the early-1990s, for example, calpain inhibitor II, a widely used calpain inhibitor, was found to inhibit the chymotrypsin-like activity of the proteasome (105). And, in the case of calpains, no inhibitor is yet able to discriminate between u- and m-calpain. Hence, the specific roles of individual calpain isoforms cannot be ascertained with inhibitors. Finally, proof that a protease degrades a

protein in a test tube is not a proof that this occurs *in vivo* because, from such studies, it cannot be known if the protein and protease are ever physically associated in the living cell and, if they are, whether the protease is appropriately regulated at that time to degrade the protein. These results described in this study are significant in that they represent one of the first attempts to specifically identify roles of proteases in living cells using specific genetic strategies. Down-regulation of an individual isoform of the calpains provides us with the first evidence for the role of the protease in a living cell. These results represent a small step toward the understanding of protein breakdown in muscle cells.

References

1. Ono, Y., Sorimachi, H. and Suzuki, K. (1999) The calpain superfamily. In *Calpain--Pharmacology and Toxicology of Calcium-Dependent Protease.* edited by Wang K.K.W. and Yuen, P.-W. 1-23.

2. Sorimachi, H., Ishiura, S., and Suzuki, K. (1997) Structure and physiological function of calpains. Biochem. J. 328:721-32.

3. Denison, S. H., Orejas, M., and Arst, H.N., Jr. (1995) Signaling of ambient pH in *Aspergillus* involves a cysteine protease. J. Biol. Chem. 270:28519-22.

4. Patrick, G.N., Zukerberg, H., Nikolic, M., de la Monte, S., Dikkes, P., and Tsai, L.H., (1999) Neurotoxicity induces cleavage of p35 to p25 by calpain. Nature 402:625-22.

5. Arthur, J.S.C., Elce, J.S., Hegadorn, C., Williams, K., and Greer, P.A. (2000) Disruption of the murine calpain small subunit gene, Capn4: calpain is essential for embryonic development but not for cell growth and division. Mole. Cell. Bio. 20:4474-81.

6. Richard, I., Broux, O., Allamand, V., Fougerousse, F., Chiannilkulchai, N., Bourg, N., Brenguier, L., Devaud, C., Pasturaud, P., and Roudaut, C., (1995) Mutations in the proteolytic enzyme calpain 3 cause limb-gridle muscular dystrophy type 2a. Cell 81:27-40.

7. Yoshimura, N., Kikuchi, T., Sasaki, T., Kitahara, A., Hatanaka, M., and Murachi, T. (1983) Two distinct Ca^{2+}proteases (calpain I and calpain II) purified concurrently by the same method from rat kidney. J. Biol. Chem. 258:8883-9.

8. Sasaki T., Kikuchi T., Yumoto N., Yoshimura N., and Murachi T. (1984) Comparative specificity and kinetic studies on porcine calpain I and calpain II with naturally occurring peptides and synthetic fluorogenic substrates. J. Biol. Chem. 259:12489-94.

9. Bartus R.T., Hayward N.J., Elliott P.J., Sawyer S.D., Baker K.L., Dean R.L., Akiyama A., Straub J.A., Harbeson S.L., Li Z., and Powers, J. (1994) Calpain inhibitor AK295 protects neurons from focal brain ischemia. Effects of postocclusion intra-arterial administration. Stroke. 25(11):2265-70.

10. Hanada, K., Tamai, M., Morinoto, S., Yamagishi, M., Ohmura, S., Sawada, J., and Tanaka, I. (1978) Studies on thiol ptotease inhibitors. PartI. Isolotion and characterisation of E64, a new thiol protease inhibitor. Agric. Biol. Chem. 42:523-8.

11. Wang K.K., Nath R., Posner A., Raser K.J., Buroker-Kilgore M., Hajimohammadreza I., Probert A. W., Marcoux 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. (1996) An alpha-mercaptoacrylic acid derivative is a selective nonpeptide cell-permeable calpain inhibitor and is neuroprotective. Proc. Natl. Acad. Sci. USA. 93:6687-92.

12. Lin G.D., Chattopadhyay D., Maki M., Wang 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. Struct. Biol. 4:539-47.

13. Elamrani N., Brustis J.J., Dourdin N., Balcerzak D., Poussard S., Cottin P., and Ducastaing A. (1995) Desmin degradation and Ca(2+)-dependent proteolysis during myoblast fusion. Biol. Cell.85:177-83.

14. Capetanaki Y., and Milner D.J. (1998) Desmin cytoskeleton in muscle integrity and function. Subcell. Biochem. 31:463-95.

15. Low, R.B. and Goldberg, A.L. (1973) Non-uniform rates of turnover of myofibrillar proteins in rat diaphragm. J. Cell Biol. 56: 590-5.

16. 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 253:192-3.

17. Goldberg, A.L. (1969a) Protein turnover in skeletal muscle. I. Protein catabolism during work-induced hypertrophy and growth induced with growth hormone. J. Biol. Chem. 244:3217-22.

18. Goldberg, A.L. (1969b) Protein turnover in skeletal muscle. II. Effects of denervation and cortisone on protein catabolism in skeletal muscles. J. Biol.Chem. 244:3223-9.

19. Huang, J., and Forsberg, N.E. (1999) Role of calpain in skeletal-muscle protein degradation. Proc. Natl. Acad. Sci. USA. 95:12100-5.

20. Nishiura, I., Tanaka, K., Yamato, S., and Murachi, T. (1978) The occurrence of an inhibitor of Ca2+-dependent neutral protease in rat liver. J. Biochem. 84:1657-9.

21. Waxman, L., and Krebs, E.G. (1978) Identification of two protenase inhibitors from bovine cardiac muscle. J. Biol. Chem. 253:5888-91.

22. Sormachi, H., Kmajoh-Ohmi, L., Emori, Y., Kawasaki, H., Ohno, S., Minami, Y., and Suzuki, K. (1989) Molecular cloning of a novel mammalian calcium-dependent protease distinct form both m- and u--types. J. Biol. Chem. 264:20106-11.

23. Ma, H., Fukiage, C., Azuma, M., and Shearer, T.R. (1998) Molecular cloning and expression of calpain Lp82 from rat lens: A splice variant of muscle p94 mRnA with insert refions deleted. Investigat. Ophthalmol. and Vis. Sci. 39:454-61.

24. Ma, H., Hata, I., Fukiage, C., Azuma, M., and Shearer, T.R. (2000) Lp85 calpain is an enzymatically active rodent-specific isozyme of lens Lp82. Curr Eye Res. 20:183-9.

25. 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 Ca(2+)-binding domain. J. Biol. Chem. 268:19476-82.

26. Lee, H.J., Sorimachi, H., Jeong, S.Y., Ishiura, S., and Suzuki, K. (1998) Molecular cloning and characterizatin of a novel tissue-specific calpain predominantly expressed in the digestive tract. Bio. Chem. 379:175-83.

27. Dear, N., Matena, K., Vingron, M., and Boehm, T. (1997) A new subfamily of vertebrate calpains lacking a calmodulin-like domain: implications for calpain regulation and evolution. Genomics 45:175-84.

28. Delaney, S.J., Hayward, K.C., Barleben, F., Fischbach, K.F., and Gabor Miklos, G.L. (1991) Molecular cloning and analysis of small optic lobes, a structural brain gene of *Drosophila melanogaster*. Proc. Natl. Acade. Sci. USA. 88:7213-18.

29. Emori, Y., and Saigo, K. (1994) Calpain localization changes during early embryonic development of Drosophila. J. Bio. Chem. 269:25173-42.

30. Waterson, R., Martin, C., Craxton, M., Huynh, C., Coulson, A., Hillier, L., Durbin, R., Green, P., Shown-keen, R., Halloran, N., Metzstein, M., Hawkins, T., Wilson, r., Berks, M., Du, Z., thomas, K., ThierryMieg, J., and Sulston, J. (1992) A survey of expressed genes in *Caenorhabditis elegans*. Nature Genetics 1:114-23.

31. Wilson, R., Alinscough, R. et al. (1994) 2.2 Mb of contiguous nucleotide sequence from chromsome III of *C. elegans*. Nature 368:32-8.

32. Goll, K.E., thompson, V.F., and Taylor, R.G., and Zalewska, T. (1992) Is calpain activity regulated by membranes and autolysis or by calcium and calpastatin? BioEssays 14:549-56.

33. Saido, T.C., Sorimachi, H., and Suzuki, K. (1994) Calpain: new perspectives in molecular diversity and physiological-pathological involvement. FASEB J. 8:814-22.

34. Yoshizawa, T., Sorimachi, H., tomioka, S., Ishiura, S., and Suzuki, K. (1995) A catalytic subuint of calpain prossesses full proteolytic activity. FEBS Letters 358:101-3.

35. Elce, J.S., Hedadorn, C., and Arthur, J.S.C. (1997) Autolysis, Ca2+ requirement, and heterodimer stability in m-calpain. J. Biol. Chem. 272:11268-75.

36. Hosfield, C.M., Elce, J.S., Davies, P.L., and Jia, Z. (1999) Crystal structure of calpain reveals the structural basis for Ca(2+)-dependent protease activity and a novel mode of enzyme activation. EMBO J. 18:6880-9.

37. Strobl S., Fernandez-Catalan C., Braun M., Huber R., Masumoto H., Nakagawa K., Irie A., Sorimachi H., Bourenkow G., Bartunik H., Suzuki K., and Bode W. (2000) The crystal structure of calcium-free human m-calpain suggests an electrostatic switch mechanism for activation by calcium. Proc. Natl. Acad. Sci. USA. 97:588-92.

38. Blanchard H., Grochulski P., Li Y., Arthur J.S., Davies P.L., Elce J.S., and Cygler M. (1997) Structure of a calpain Ca(2+)-binding domain reveals a novel EF-hand and Ca(2+)-induced conformational changes. Nat. Struct. Biol. 4:532-8.

39. Lin G.D., Chattopadhyay D., Maki M., Wang 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. Struct. Biol. 4:539-47.

40. Yoshizawa T., Sorimachi H., Tomioka S., Ishiura S., and Suzuki K. (1995) Calpain dissociates into subunits in the presence of calcium ions. Biochem. Biophys. Res. Commun. 208:376-83.

41. Zhang W., and Mellgren R.L. (1996) Calpain Subunits Remain Associated during Catalysis. Biochem. Biophys. Res. Commun. 227:890-6.

42. Elce J.S., Davies P.L., Hegadorn C., Maurice D.H., and Arthur J.S. (1997) The effects of truncations of the small subunit on m-calpain activity and heterodimer formation. Biochem. J. 326:31-8.

43. Johnson, G.V., and Guttmann R.P. (1997) Calpains: intact and active? Bioessays 19:1011-8.

44. Saido T.C., Nagao S., Shiramine M., Tsukaguchi M., Yoshizawa T., Sorimachi H., Ito H., Tsuchiya T., Kawashima S., and Suzuki K. (1994) Distinct kinetics of subunit autolysis in mammalian m-calpain activation. FEBS Lett. 346:263-7.

45. Tompa P., Baki A., Schad E., and Friedrich P. (1996) The calpain cascade. Mu-calpain activates m-calpain. J. Biol. Chem. 271:33161-4.

46. Sultan, K.R., Dittrich, B.T., and Pette, D. (2000) Calpain activity in fast, slow, transforming, and regenerating skeletal muscles of rat. Am. J. Physiol. Cell Physiol. 279:C639-47.

47. Thompson, V.F., Lawson, K., and Goll, D.E. (2000) Effect of u-calpain on m-calpain. Biochem. Biophys. Res. Commun. 267:495-9.

48. Melloni E., Averna M., Salamino F., Sparatore B., Minafra R., and Pontremoli S. (2000) Acyl-CoA-binding protein is a potent m-calpain activator. J. Biol. Chem. 275:82-6.

49. 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. 249:583-8.

50. Richard I., Broux O., Allamand V., Fougerousse F., Chiannilkulchai N., Bourg N., Brenguier L., Devaud C., Pasturaud P., Roudaut C., et al (1995) Mutations in the proteolytic enzyme calpain 3 cause limb-girdle muscular dystrophy type-2A. Cell 81:27-40.

51. Tagawa K., Taya C., Hayashi Y., Nakagawa M., Ono Y., Fukuda R., Karasuyama H., Toyama-Sorimachi N., Katsui Y., Hata S., Ishiura S., Nonaka I., Seyama Y., Arahata K., Yonekawa H., Sorimachi H., and Suzuki K. (2000) Myopathy phenotype of transgenic mice expressing active site-mutated inactive p94 skeletal muscle-specific calpain, the gene product responsible for limb girdle muscular dystrophy type 2A. Hum. Mole. Genet. 9:1393-402.

52. Yoshikawa Y., Mukai H., Hino F., Asada K., and Kato I. (2000) Isolation of two novel genes, down-regulated in gastric cancer. Jpn. J. Cancer Res. 91:459-63.

53. Liu K., Li L., and Cohen S.N. (2000) Antisense RNA-mediated deficiency of the calpain protease, nCL-4, in NIH3T3 cells is associated with neoplastic transformation and tumorigenesis. J. Biol. Chem. 275:31093-8.

54. Zimmerman U.J., Boring L., Pak J.H., Mukerjee N., and Wang K.K. (2000) The calpain small subunit gene is essential: its inactivation results in embryonic lethality. IUBMB Life. 50:63-8.

55. Saatman K.E., Zhang C., Bartus R.T., and McIntosh T.K. (2000) Behavioral efficacy of post-traumatic calpain inhibition is not accompanied by reduced spectrin proteolysis, cortical lesion, or apoptosis. J. Cereb. Blood Flow Metab. 20:66-73.

56. Shields D.C., Schaecher K.E., Hogan E.L., and Banik N.L. (2000) Calpain activity and expression increased in activated glial and inflammatory cells in penumbra of spinal cord injury lesion. J. Neurosci. Res. 61:146-50.

57. Saatman K.E., Murai H., Bartus R.T., Smith D.H., Hayward N.J., Perri B.R., and McIntosh T.K (1996) Calpain inhibitor AK295 attenuates motor and cognitive deficits following experimental brain injury in the rat. Proc. Natl. Acad. Sci. USA. 93:3428-33.

58. Ray S.K., Matzelle D.C., Wilford G.G., Hogan E.L., and Banik N.L. (2000) E-64-d prevents both calpain upregulation and apoptosis in the lesion and penumbra following spinal cord injury in rats. Brain Res. 867:80-9.

59. Ray S.K., Fidan M., Nowak M.W., Wilford G.G., Hogan E.L., and Banik N.L. (2000) Oxidative stress and Ca2+ influx upregulate calpain and induce apoptosis in PC12 cells. Brain Res. 852:326-34.

60. Maruyama K., Usami M., Kametani F., Tomita T., Iwatsubo T., Saido T.C., Mori H., and Ishiura S. (2000) Molecular interactions between presenilin and calpain: inhibition of m-calpain protease activity by presenilin-1, 2 and cleavage of presenilin-1 by m-, mu-calpain. Int. J. Mol. Med. 5:269-73.

61. Nath R., Davis M., Probert A.W., Kupina N.C., Ren X., Schielke G.P., and Wang K.K. (2000) Processing of cdk5 activator p35 to its truncated form (p25) by calpain in acutely injured neuronal cells. Biochem. Biophys. Res. Commun. 274:16-21.

62. Lee M.S., Kwon Y.T., Li M., Peng J., Friedlander R.M., and Tsai L.H. (2000) Neurotoxicity induces cleavage of p35 to p25 by calpain. Nature 405:360-4.

63. Kusakawa G., Saito T., Onuki R., Ishiguro K., Kishimoto T., and Hisanaga S. (2000) Calpain-dependent proteolytic cleavage of the p35 cyclin-dependent kinase 5 activator to p25. J. Biol. Chem. 275:17166-72.

64. Touyarot K., Poussard S., Verret C., Aragon B., Cottin P., Nogues X., and Micheau J. (2000) Calpain-PKC inter-relations in mouse hippocampus: a biochemical approach. Neurochem. Res. 25:781-90.

65. Atencio I.A., Ramachandra M., Shabram P., and Demers G.W. (2000) Calpain inhibitor 1 activates p53-dependent apoptosis in tumor cell lines. Cell Growth Differ. 11:247-53.

66. Ray S.K., Matzelle D.C., Wilford G.G., Hogan E.L., and Banik N.L. (2000) E-64-d prevents both calpain upregulation and apoptosis in the lesion and penumbra following spinal cord injury in rats. Brain Res. 867:80-9.

67. Zhu D.M., and Uckun F.M. (2000) Calpain inhibitor II induces caspase-dependent apoptosis in human acute lymphoblastic leukemia and non-Hodgkin's lymphoma cells as well as some solid tumor cells. Clin. Cancer Res. 6:2456-63.

68. Rami A., Agarwal R., Botez G., and Winckler J. (2000) mu-Calpain activation, DNA fragmentation, and synergistic effects of caspase and calpain inhibitors in protecting hippocampal neurons from ischemic damage. Brain Res. 866:299-312.

69. Lankiewicz S., Marc Luetjens C., Truc Bui N., Krohn A.J., Poppe M., Cole G.M., Saido T.C., and Prehn J.H. (2000) Activation of calpain I converts excitotoxic neuron death into a caspase-independent cell death. J. Biol. Chem. 275:17064-71.

70. Barnes T.M., and Hodgkin J. (1996) The tra-3 sex determination gene of Caenorhabditis elegans encodes a member of the calpain regulatory protease family. EMBO J. 15:4477-84.

71. Nelson W.J., and Traub P. (1983) Proteolysis of vimentin and desmin by the Ca2+-activated proteinase specific for these intermediate filament proteins. Mol. Cell. Biol. 3:1146-56.

72. Dayton, W.R., Goll, D.E., Stromer, M.H., Reville, W.J., Zeece, M.g., and Robson, R.M. (1975) Some properties of a Ca2+-activated protease that may be involvedin myofibrillar protein turnover. In *Cold Spring Harbor Conferences on Cell Proliferation*, vol. 2, *Proteases and Biological Control*, eds. Reich, E., Rifkin, D.B., and Shaw E., 551-7. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

73. Goll D.E., Thompson V.F., Taylor R.G., and Christiansen J.A. (1992) Role of the calpain system in muscle growth. Biochimie. 74:225-37.

74. Spencer M.J., and Tidball J.G. (1992) Calpain concentration is elevated although net calcium-dependent proteolysis is suppressed in dystrophin-deficient muscle. Exp. Cell. Res. 203:107-14.

75. Li Z., Mericskay M., Agbulut O., Butler-Browne G., Carlsson L., Thornell L.E., Babinet C., and Paulin D. (1997) Desmin is essential for the tensile strength and integrity of myofibrils but not for myogenic commitment, differentiation, and fusion of skeletal muscle. J. Cell. Biol. 139:129-44.

76. Groll M., Ditzel L., Lowe J., Stock D., Bochtler M., Bartunik H.D., and Huber R. (1997) Structure of 20S proteasome from yeast at 2.4 A resolution. Nature 386:463-71.

77. 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. 271:26690-7.

78. Forsberg N.E., Ilian M.A, Ali-Bar A., Cheeke 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. 67:3313-21.

79. Bardsley R.G., Allcock S.M., Dawson J.M., Dumelow N.W., Higgins J.A., Lasslett Y.V., Lockley A.K., Parr T., and Buttery P.J. (1992) Effect of beta-agonists on expression of calpain and calpastatin activity in skeletal muscle. Biochimie. 74:267-73.

80. Parr T., Bardsley R.G., Gilmour R.S., and Buttery P.J. (1992) Changes in calpain and calpastatin mRNA induced by beta-adrenergic stimulation of bovine skeletal muscle. Eur. J. Biochem. 208:333-9.

81. Kretchmar D.H., Hathaway M.R., Epley R.J., and Dayton W.R. (1989) In vivo effect of a beta-adrenergic agonist on activity of calcium-dependent proteinases, their specific inhibitor, and cathepsins B and H in skeletal muscle. Arch. Biochem. Biophys. 275:228-35.

82. Milner D.J., Mavroidis M., Weisleder N., and Capetanaki Y. (2000) Desmin cytoskeleton linked to muscle mitochondrial distribution and respiratory function. J. Cell. Biol. 150:1283-98.

83. Reid W.D., Huang J., Bryson S., Walker D.C., and Belcastro A.N. (1994) Diaphragm injury and myofibrillar structure induced by resistive loading. J. Appl. Physiol. 76:176-84.

84. Barnoy S., Glaser T., and Kosower N.S. (1998) The calpain-calpastatin system and protein degradation in fusing myoblasts. Biochim. Biophys. Acta. 1402:52-60.

85. Warren R.H. (1997) TGF-alpha-induced breakdown of stress fibers and degradation of tropomyosin in NRK cells is blocked by a proteasome inhibitor. Exp. Cell. Res. 236:294-303.

86. Gao W.D., Atar D., Liu Y., Perez N.G., Murphy A.M., and Marban E. (1997) Role of troponin I proteolysis in the pathogenesis of stunned myocardium. Circ. Res. 80:393-9.

87. Ilian M.A., and Forsberg N.E. (1992) Gene expression of calpains and their specific endogenous inhibitor, calpastatin, in skeletal muscle of fed and fasted rabbits. Biochem J. 287:163-71.

88. Hata A., Ohno S., and Suzuki K. (1992) Transcriptional activation of the gene for the large subunit of human m-calpain by 12-o-tetradecanoyl-phorbol-13-acetate. FEBS Lett. 304:241-4.

89. Zhang J., Patel J.M., and Block E.R. (1998) Hypoxia-specific upregulation of calpain activity and gene expression in pulmonary artery endothelial cells. Am. J. Physiol. 275:L461-8.

90. Rami A., Agarwal R., Botez G., and Winckler J. (2000) mu-Calpain activation, DNA fragmentation, and synergistic effects of caspase and calpain inhibitors in protecting hippocampal neurons from ischemic damage. Brain Res. 866:299-312.

91. Farah C.S., and Reinach F.C. 91995) The troponin complex and regulation of muscle contraction. FASEB J. 9:755-67.

92. Uehara Y., Mori C., Noda T., Shiota K., and Kitamura N. (2000) Rescue of embryonic lethality in hepatocyte growth factor/scatter factor knockout mice. Genesis. 27:99-103.

93. Goebel H.H., and Warlo I. (2000) Gene-related protein surplus myopathies. Mol. Genet. Metab. 71:267-75.

94. Goebel H.H., and Warlo I.A. (2000) Progress in desmin-related myopathies. J. Child. Neurol. 2000:565-72.

95. Sugawara M., Kato K., Komatsu M., Wada C., Kawamura K., Shindo P.S., Yoshioka P.N., Tanaka K., Watanabe S., and Toyoshima I. (2000) A novel de novo mutation in the desmin gene causes desmin myopathy with toxic aggregates. Neurology. 55:986-90.

96. Arthur G.D., Booker T.S., and Belcastro A.N. (1999) Exercise promotes a subcellular redistribution of calcium-stimulated protease activity in striated muscle. Can. J. Physiol. Pharmacol. 77:42-7.

97. Schumacher P.A., Siman R.G., and Fehlings M.G. (2000) Pretreatment with calpain inhibitor CEP-4143 inhibits calpain I activation and cytoskeletal degradation, improves neurological function, and enhances axonal survival after traumatic spinal cord injury. J. Neurochem. 74:1646-55.

98. Schumacher P.A., Eubanks J.H., and Fehlings M.G. (1999) Increased calpain I-mediated proteolysis, and preferential loss of dephosphorylated NF200, following traumatic spinal cord injury. Neuroscience. 91:733-44.

99. Bartus R.T., Hayward N.J., Elliott P.J., Sawyer S.D., Baker K.L., Dean R.L., Akiyama A., Straub J.A., Harbeson S.L., Li Z., et al. (1994) Calpain inhibitor AK295 protects neurons from focal brain ischemia. Effects of postocclusion intra-arterial administration. Stroke. 25:2265-70.

100. Jiang Q., and Stys P.K. (2000) Calpain inhibitors confer biochemical, but not electrophysiological, protection against anoxia in rat optic nerves. J. Neurochem. 74:2101-7.

101. Saatman K.E., Zhang C., Bartus R.T., and McIntosh T.K. (2000) Behavioral efficacy of posttraumatic calpain inhibition is not accompanied by reduced spectrin proteolysis, cortical lesion, or apoptosis. J. Cereb. Blood Flow Metab. 20:66-73.

102. Harriman J.F., Waters-Williams S., Chu D.L., Powers J.C., and Schnellmann R.G. (2000) Efficacy of novel calpain inhibitors in preventing renal cell death. J. Pharmacol. Exp. Ther. 294:1083-7.

103. Gallagher, S., Winston, S.E., Fuller, S.A., and Hurrell, J.G.R. (2000) Immunoblotting and immunodetection. In *Current Protocols of Molecular Biology*, 10.8.1-10.8.21. eds. by Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. John Wiley & Sons, Inc.

104. Samaha F.J., and Quinlan J.G. Dystrophinopathies: clarification and complication. J. Child. Neurol. 11:13-20.

105. Figueiredo-Pereira M.E., Banik N., and Wilk S. (1994) Comparison of the effect of calpain inhibitors on two extralysosomal proteinases: the multicatalytic proteinase complex and m-calpain. J. Neurochem. 62:1989-94.

Chapter III

Development of a Skeletal Muscle-Specific Ecdysone-Inducible Expression System

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Abstract

The goal of this study was to develop a muscle-specific inducible gene expression system in order to allow us to assess calpain function in skeletal muscle. Others, including members of our laboratory, have developed muscle lines such as these. However, our goal was to develop a method which may be applied *in vivo*. Hence, we chose the ecdysone inducible system as others have used this system to express exogenous genes in transgenic animals. The limitation of the ecdysone system was its constitutive expression in all tissues. Hence, its application *in vivo* would result in expression of a cloned gene in all tissues. To target its expression to muscle, we removed the CMV promoter and replaced it with a skeletal muscle-specific alpha-actin promoter so that the regulatory features of the system would be expressed in skeletal muscle only. We transfected our newly-designed expression system into L8 muscle cells and determined that reporter gene activity was induced by ponasterone A (inducer) in myotubes, a differentiated muscle phenotype, but not in myoblasts (undifferentiated cells). This proved the validity of the concept of an inducible muscle-specific expression system. We then determined that beta-galactosidase expression was dependent upon the duration of ponasterone A dose and duration of exposure to inducer. This creates potential to regulate both the level of expression and duration of expression of a cloned gene. In the course of the work, we discovered an extra 122-bp fragment in the expression plasmid and removed this with the hope that this manipulation might enhance the inducibility of the ecdysone system in muscle. However, this fragment was found to not affect the ability of ponasterone A to regulate beta-galactosidase expression.

Keywords: ecdysone inducible expression system, a-actin promoter, rat, B-gal assay, reporter gene

Introduction

In the past decade, several inducible gene expression systems have been developed for mammalian cells. These include the LacSwitch, ecdysone (1) and Tet-on/Tet-off systems. The advantage of these systems is that they allow precise control of gene expression at a specific time. Limitations are leakiness (i.e., expression in the absence of inducer) and lack of tissue-specificity.

In earlier studies we developed all cell lines in which we could expression calpain in skeletal muscle (2). These studies were based on the LacSwitch expression system which, unfortunately, does not allow application *in vivo*. In this study, we decided to investigate potential for the ecdysone system in muscle cells as others have demonstrated its potential in transgenic mice. This study was designed to build on the previous chapter to allow us to exactally assess calpain function in living muscle.

The ecdysone system (1), available from Invitrogen (Invitrogen, Carlsbad, CA), causes widespread expression of a cloned gene when animals are treated with inducer. For our needs, we had hoped to develop an expression system which would function only in skeletal muscle. To create skeletal muscle-specificity, we replaced the constitutively-active CMV promoter of the ecdysone regulatory plasmid with the rat skeletal alpha-actin promoter (3) with a hypothesis that this strategy would result in inducible expression of a cloned gene in skeletal muscle only.

Materials and Methods

Preparation of Plasmids

Preparation of pAP: Based on the restriction site analysis of pVgRXR (Figure 3.1), two PstI sites were identified on either side of the CMV promoter. We digested the plasmid with PstI to excise this fragment. PstI was also chosen since the alpha-actin fragment which we later subcloned into this site lacked a PstI site.

A BspHI site was inserted as a cohesive end to join the zeocin-resistance gene and skeletal alpha-actin promoter together. Therefore, the final ligation required ligation of three fragments to subclone the actin promoter fragment from pJB5 (3) into pVgRXR. To accomplish this, the zeocin resistance fragment and alpha-actin promoter were amplified by PCR and were then double-digested with PstI and BspHI. pVgRXR was digested with PstI as well. After purification of the fragments, they were ligated overnight to create a new vector which we designated as pAP (actin promoter). In fact, the production of pAP was not simple. Several intermediate steps were involved as shown in Figure 3.2. First, the zeocin resistant gene was ligated into pCR2.1 (Figure 3.1, vector pCR2.1Z) and the alpha-actin promoter was ligated into pCR2.1AP (Figure 3.2). The new vector was designated as pCR2.1AP (Figure 3.2). Finally, the ligated fragment consisting of the zeocin: alpha-actin fragment was "cut" from pCR2.1A by PstI and ligated into PstI-digested pVgRXR to create the pAP vector. Correct orientation and sequence of pAP were verified by sequencing in OSU's Center for Gene Research.

Plasmid Amplification: Plasmids were used to transform self-made E. Coli DH5α strain according to standard methods (4). Transfected bacteria were spread on LB/agar plates containing appropriate antibiotics: (Ampicillin at 100 ug/ml or Zeocin at (100 ug/ml) for ampicillin- and zeocin-resistant colonies, respectively. Antibiotic-resistant colonies were gown in large-scale and plasmid DNA was prepared from cultures using the NucleoBond

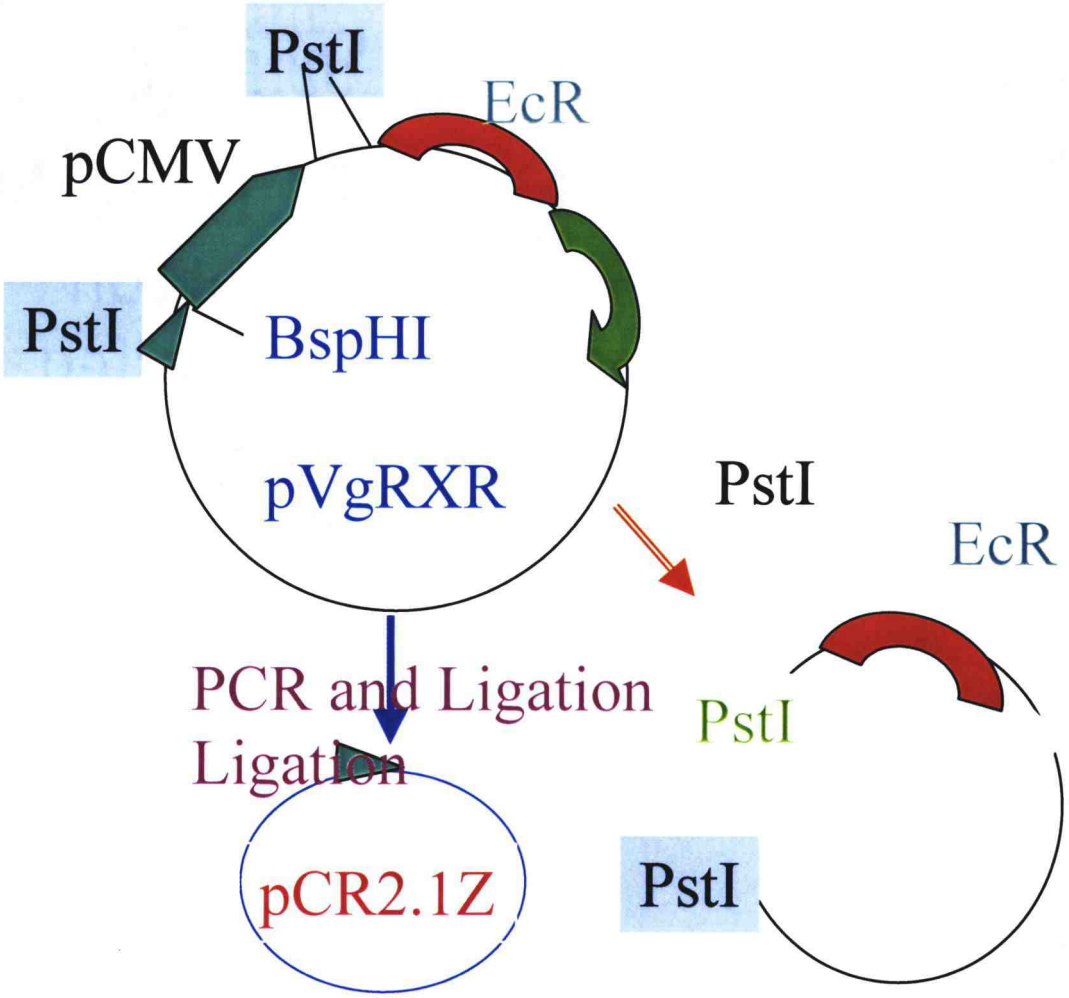


Figure 3.1. Schematic diagram showing the positions of PstI restriction sites, zeocin resistant gene (solid triangle), cytomegalovirus promoter pCMV (rocket shape) and ecdysone receptor EcR (wide rainbow) in pVgRXR. Zeocin resistant gene (PstI/BspHI fragment) was subcloned into pCR2.1 to creat a new vector designated as pCR2.1Z. The restriction site BspHI shows where it was inserted (between zeocin and a-actin promoter). After PstI digestion, pCMV was removed from the pVgRXR sequence.

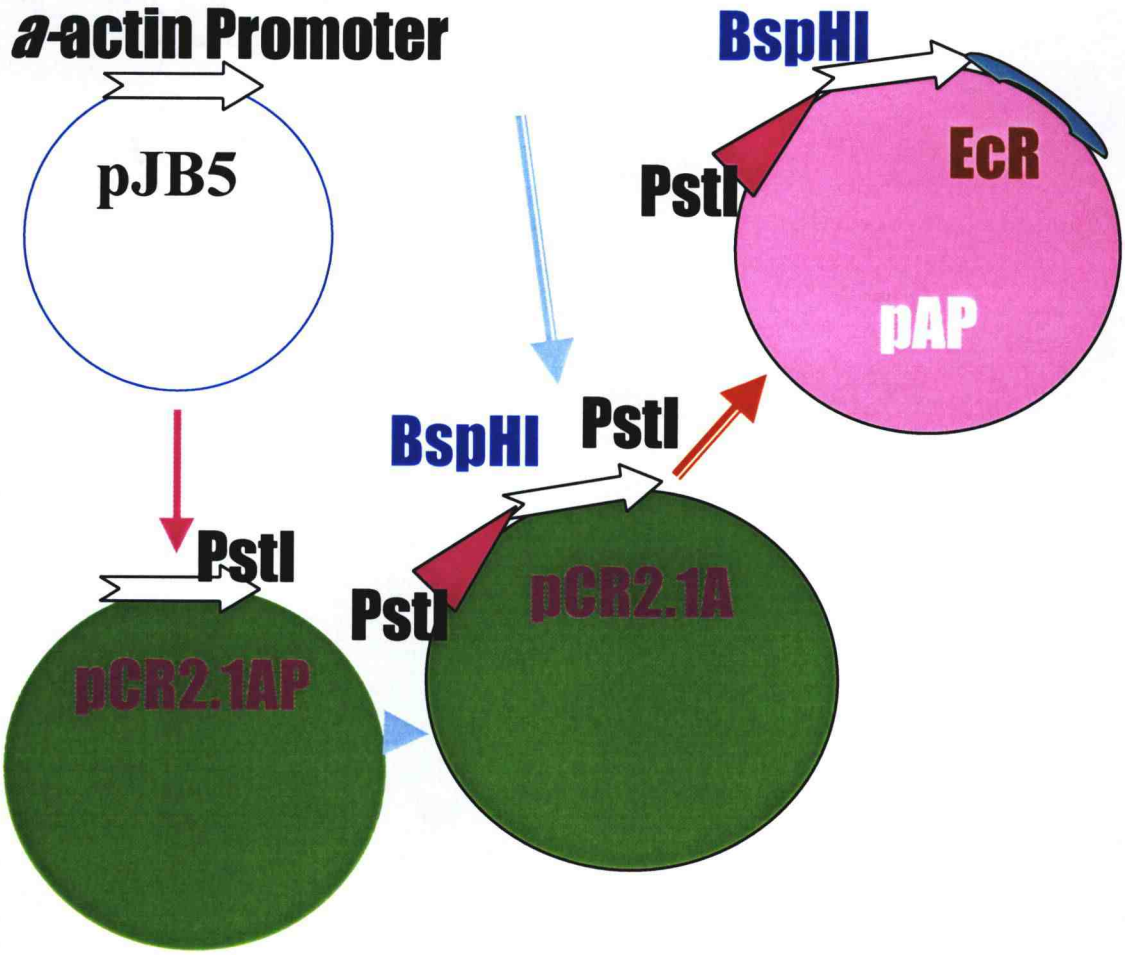


Figure 3.2. Schematic diagram showing the preparation of pAP. The α-actin promoter (from pJB5) was subcloned into pCR2.1 (the new vector is pCR2.1AP). Then the a-actin promoter was subcloned into pCR2.1Z for fusing with the zeocin resistant gene. The new vector was designated as pCR2.1A. Finally, the PstI fragment containing the fused zeocin resistant gene and α-actin promoter was inserted into PstI-digested pVgRXR sequence to create pAP. This plasmid thereby placed expression of the ecdysone receptor (EcR) under control of the α-actin promoter. Open arrow: α-actin promoter; solid triangle:zeocin resistant gene.

Plasmid Maxi Kit (Clontech, Palo Alto, CA) and the concentration of recovered plasmid DNA was determined by comparison to lambda/HindIII standards (Gibco) on a 1% agarose TAE gel.

Cell Culture

Rat L8 myoblasts were obtained from American Type Culture Collection (ATCC: Manassas, VA) and cultured in DMEM containing either 10% fetal bovine serum (FBS for proliferation) or 2% horse serum (HS for differentiation). DMEM (low glucose) was obtained from Gibco (Gaithersburg, MD). Media contained 3.7 g/L of sodium bicarbonate and 100 U/ml penicillin-streptomycin. FBS were obtained from Hyclone (Logan, UT). When selection was applied to cells after transfection, G418 (Gibco; 400 ug/ml) and Zeocin (Invitrogen, 200 ug/ml) were added to culture media. Media was replaced every 48 hours. Myoblasts were induced to differentiate into myotubes when they reached 90% confluency.

To passage cells, medium was removed and a solution of trypsin (2.5 mg/ml; Gibco) was added to the culture dish. Cells were placed in a test tube and 1% FBS medium was added to inactivate trypsin (Gibco). Suspended cells were centrifuged at 1000 rpm for 4 min and the cell pellet was resuspended in DMEM. For storage, cells were placed in vials with a 9:1 ratio of FBS medium to DMSO (Sigma, St. Louis, MO) and stored at –80 degrees C. For subculture, the cells were diluted into an appropriate volume of FBS/DMEM medium and evenly distributed on culture dishes.

Transfection of L8 Cells

Establishment of stable cell lines: LipofectAMINE reagent (Gibco) was used to co-transfect the regulatory vectors (pAP and pIND/LacZ) into rat L8 myoblasts. The transfection procedure was based on the manufacturer's guidelines with some

modifications as needed by the L8 cell line. Briefly, the day before transfection, we trypsinized myoblasts (at 80% confluency) and redistributed them onto three 100-mm plates so that they were more than 50% confluent at the day of transfection. At the time of plating and during transfection, antibiotics were not added to culture media. At transfection, we diluted 2 ug of pAP and pIND/LacZ into 300 ul of serum-free DMEM. Similarly, 10 ul of LipofectAMINE reagent was diluted into this sample and allowed to sit at room temperature for 15 minutes. This allowed complex formation between the plasmids and transfection reagent. During this time, we replaced medium from the cells with 5 ml serum-free DMEM after which the transfection solution was added to cultures and mixed gently. The plates of cells were then incubated for 3 hr at 37°C in a 5% CO_2 incubator. After 3 hours, we replaced the medium with fresh complete (DMEM containing 10% FBS) medium and incubated the plates for 1 day. After this, selection medium was applied which contained both G418 and Zeocin. After 24 hours, the cells were removed from their plates by trypsinization and the cells were redistributed and diluted onto other plates in selection media.

Transient transfection: Transient transfection was finished by using LipofectAmine (Gibco) reagent according to the producer's protocol. Three ug of high-quality plasmid (pIND/LacZ and pVgRXR, or pIND/LacZ and pVgD) were used for co-transfecting rat L8 cells in each 60 mm cell culture dish when cells were 50-70% confluent. After 6 hr incubation, the transfection medium was withdrawn and normal medium (with 10% FBS) was applied for further 12 hr-recovery incubation. Then, 5 uM ponasterone A was added to the medium and the cells were incubated further for 24 hr. The cells are harvested for β-galactosidase activity assay. For control plates there was no plasmids added to the transfection reagent and the other procedures were the same.

Beta-galactosidase Assays

Beta-galactosidase staining assay: To test for the efficacy of expression, we assayed beta-galactosidase (B-Gal) expression following induction by addition of ponasterone A to culture media. A B-Gal staining kit (Invitrogen) was utilized to visualize the

expression of the LacZ gene product in stable-transfected myotubes containing pVgAP and pIND/LacZ. The manufacturer's protocol for assessing B-Gal activity was followed. Photographs of B-Gal expression were taken 4 hours after addition of staining reagents.

High-sensitivity beta-galactosidase activity assay: In order to quantitatively measure the inducibility of the skeletal muscle-specific expression system, a high-sensitivity B-Gal activity assay was utilized. This assay was performed according to the protocol of the manufacturer (Strategene, La Jolla, CA) with the following modifications. Five to ten ul of crude cell extract were applied to 900 ul of 1X CPRG substrate. For the assay, an incubation of 37°C was used but duration varied, depending upon the rate of color development. And an OD reading at 580 nm was used for all measurements. Protein concentration was measured using Bio-Rad (Hercules, CA) Protein Assay according to the manufacturer's protocol and B-Gal activity was expressed as a proportion of cellular protein.

Statistical Analysis

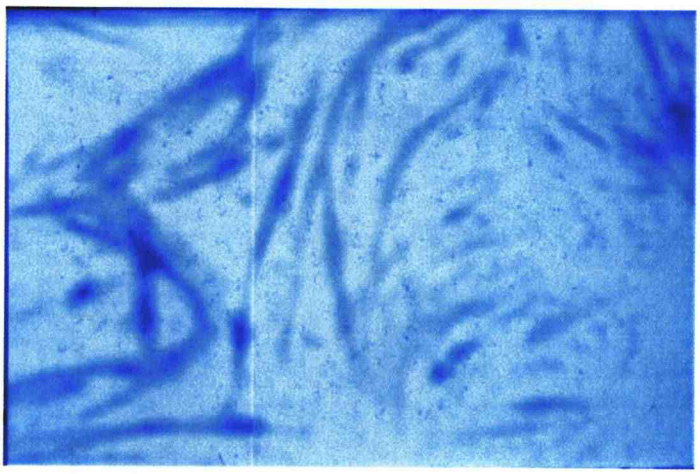
All experiments for quantitative assays were performed at least three times with three replicates within each study. The plots of the data were made with NCSS (Kaysville, Utah). Analysis of variance (ANOVA) was used to determine if differences existed among treatment means. A Student-Neuman-Keul multiple range test was used to evaluate individual treatment differences. A significance level of 5% was adopted for all comparisons.

Results and Discussion

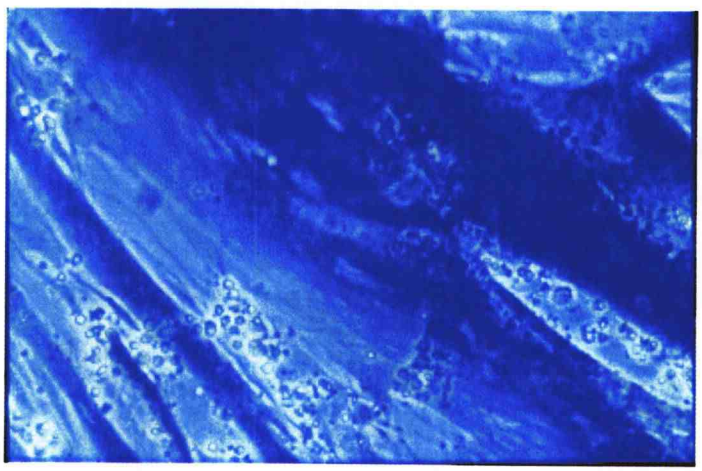
Rat L8 myoblasts were transfected with pAP and reporter plasmid (pIND/LacZ) and stable transfectants which were resistant to both zeocin and G418 were selected and expanded. Once stable cell lines were identified, they were stored frozen with DMSO in liquid nitrogen or at $-80^{o}C$. As designed, the plasmid expression system should express β-galactosidase in differentiated muscle cells but not in the undifferentiated myoblasts (undifferentiated muscle progenitor cells).

To test the efficacy of this new expression system, we cultured a stable cell line as myoblasts (undifferentiated phenotype) and as myotubes (differentiated phenotype). Ponasterone A was added to culture media and effects of this addition on cell B-galactosidase expression were assessed. Ponasterone A induced expression of B-Gal in myotubes but not in myoblasts (Figure 3.3). In stable myoblasts transfected by pAP and pIND/LacZ, there was a small reporter gene activity in induced myoblasts compared to the non-induced myoblast control (Figure 3.4). This induction may be due to a small percent of myoblasts which may be undergoing spontaneous differentiation. Comparing the absolute β-galactosidase activity (u/mg), however, between treated myoblast (Figure 3.4) and treated myotubes (Figure 3.5 and 3.6), we concluded that the differentiation in the induced myoblast groups was very low. And, the leakiness of skeletal muscle-specific expression system in myoblasts was also very low.

Ponasterone A's effects on β-gal expression were also dose-dependent (Figures 3.5 and 3.6). As ponasterone A concentration was increased from 0 to 24uM, B-Gal concentration in the myotubes was also increased. At 0 uM ponasterone A, virtually no β-Gal activity was detectable indicating that the "leakiness" of the modified ecdysone system in myotubes was very low. After 48 hours of induction (Figure 3.6) with 24 uM ponasterone A, the activity of the reporter gene (B-Gal) was 15-fold higher than control. Similarly, the longer the induction, the higher was reporter gene expression (Figures 3.5



A



B

Figure 3.3. Effects of ponasterone A (12uM; Panel A and 24uM; Panel B) on B-Gal staining in myoblasts and myotubes. Cells were assessed following 24 hours of induction.

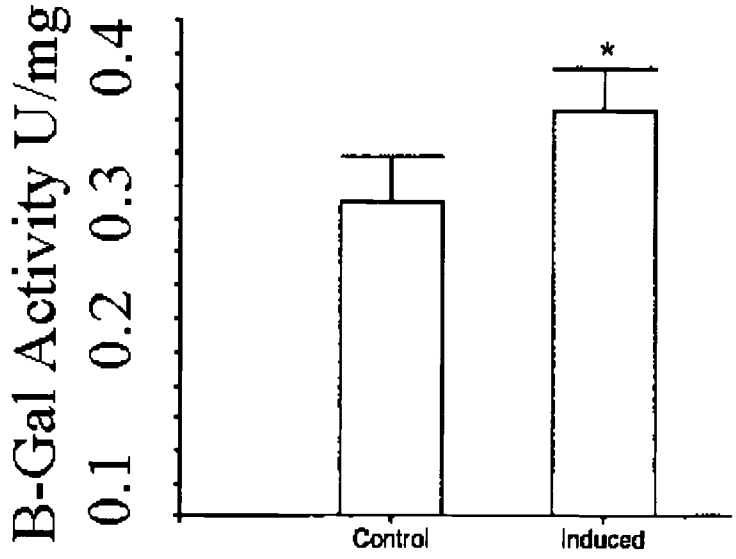


Figure 3.4. The inducibility of skeletal muscle-specific expression system in myoblasts. Stable rat L8 myoblasts transfected by pVgAP and pIND/LacZ was incubated with 16 uM ponasterone A or 16 uL 100% ethanol (control) for 24 hr and B-Gal activity was assessed.

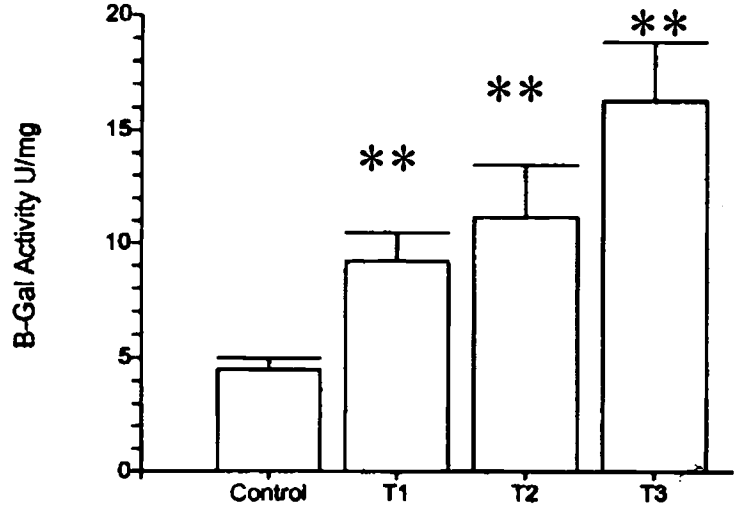


Figure 3.5. Effects of ponasterone A concentration on B-galactosidase expression in transfected L8 myotubes. Cells were exposed to ponasterone A for 24 hr. The bars represent the means+SE. 100% ethanol (vehicle) was added to control. T1, T2 and T3 represent 12 uM, 16 uM and 24 uM ponasterone A added to the myotubes. ** designates that the B-Gal activity in treated cells was significantly higher than that in the control cells ($P<0.01$).

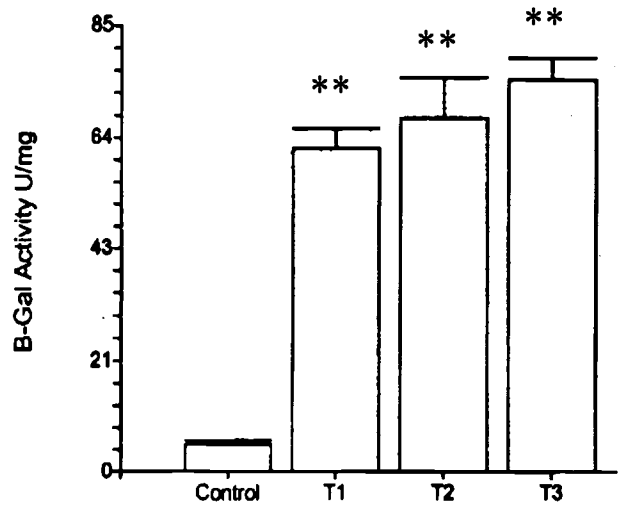


Figure 3.6. Effects of ponasterone A concentration on B-galactosidase expression in transfected L8 myotubes. Cells were exposed to ponasterone A for 48hr. The bars represent SE. 100% ethanol (vehicle) was added to control. T1, T2 and T3 represent 12 uM, 16 uM and 24 uM ponasterone A added to the myotubes. ** designates that the treatment differred from the control ($P<0.01$).

and 3.6). For example, with the same concentration of ponasterone A, B-Gal activity was 4.7-fold higher after 48 hours of induction compared to 24 hours of induction.

Inducibility and "leakiness" are important issues in designing transgenic cells and animals to study functions of individual genes. Without high levels of expression or with "leakiness", it is difficult to correctly assay protein function. By these two criteria, our redesigned muscle expression system offers potential to study muscle-specific functions of proteins.

During conduct of this study, we discovered that the Invitrogen pVgRXR plasmid contained an extra sequence which was not reported in product literature. This sequence consisted of an extra 122 bp located between the CMV and ecdysone promoter fragments and was flanked by two PstI sites. We determined, using a BLAST search (4), that there was no purpose for the extra nucleotide sequence and that the sequence was not relevant to pVgRXR function. We hypothesized that the deletion of this fragment might improve inducibility of the ecdysone system.

After deletion of the PstI sequence (using standard molecular techniques), we prepared a new expression vector which we designated as pVgD (Deleted). We then transiently transfected L8 myoblasts and assessed B-Gal expression in pAP- and pVgD-transfected cells (pIND/LacZ was cotransfected). Results are shown in Figure 2.7. In this study, we found that the deletion of this fragment had no effect ($P>.05$) on the expression of B-Gal in myotubes and conclude that the fragment did not influence efficacy of the expression system.

Transfection of cultured muscle cells with an ecdysone system in which expression of one component of the ecdysone regulation of expression was placed under control of the alpha-actin promoter efficiently resulted in a muscle-specific inducible system with a low level of "leakiness". This was indicated by the expression of B-Gal in myotubes but not in myoblasts and by the low level of expression in cells which were not treated with ponasterone A. The limitation of the system is that the level of induction caused by

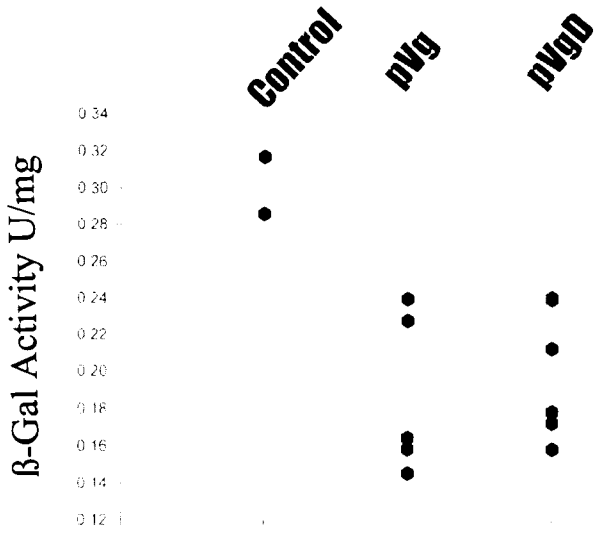


Figure 3.7. Comparison of the inducibility of pVgRXR and pVgD. Myoblasts were transiently transfected with plasmids pVgRXR or pVgD (in which a 122-bp fragment was deleted) and their responses to ponasterone A were assessed following 24 hours of exposure. pIND/LacZ was also simutaneously transfected with pVgRXR and pVgD to complete the transfection system for each experiment.

ponasterone A was not very high. Depending upon the application, this could present a problem in application, particularly when high levels of expression of a cloned gene are necessary. To increase expression, some modifications of this system might be attempted. For example, RXR ligand may be co-transfected so that it does not become limiting when ponasterone A is added to culture media (5). In addition, the side effects of over-expressing RXR in cells must be considered.

In this study, we have successfully developed a method for muscle-specific inducible gene expression based on the ecdysone expression system. It may be used to study a wide range of functions of proteins in muscle and could, potentially, be applied *in vivo*. The strategy may be used to modulate gene expression in domestic animals to enhance skeletal muscle growth or in humans to treat the genetic muscle diseases. Other tissue-specific versions of this system could be designed simply be replaced the muscle alpha-actin promoter with other tissue-specific promoter sequences. Hence, this approach has wide applicability.

Acknowledgements

The authors are grateful to Yung-Hae Kim and Yoji Ueda for their critical input. We also thank Dr. Graham Bulfield (University of London, UK) for generously providing pJB5 which contained the rat alpha-actin promoter. The research was supported by USDA grant 97-32605-2604.

References

1. No, D., Yao, T.P., and Evans, R.M. (1996). Ecdysone-inducible gene expression in mammalian cells and transgenic mice. Proc. Natl. Acad. Sci. USA. 93, 3346-3351.

2. Huang J., and Forsberg N.E. (1998) Role of calpain in skeletal-muscle protein degradation. Proc. Natl. Acad. Sci. USA. 95:12100-5.

3. Asante, E.A., Boswell, J.M., Burt, D.W., and Bulfield, G. (1994). Tissue specific expression of an α-skeletal actin-lacZ fusion gene during development in transgenic mice. Transgenic Res. 3, 59-66.

4. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. (1990) Basic local alignment search tool. J. Mol. Biol. 215:403-10.

5. Saez E., Nelson M.C., Eshelman B., Banayo E., Koder A., Cho G.J., and Evans R.M. (2000) Identification of ligands and coligands for the ecdysone-regulated gene switch. Proc. Natl. Acad. Sci. USA. 97:14512-7.

Chapter IV

Overall Summary

In order to study the role of calpain on skeletal muscle total protein degradation, we developed several strategies to down-regulate endogenous calpain activity in skeletal muscle cells (myotubes). These included over-expression of antisense u-calpain (AnsL), dominant negative u-calpain (DN-u-CL), antisense 30K subunit (AnsS) and fused antisense u-calpain/30K. For the purpose of distinguishing the DN-u-calpain and endogenous u-calpain (there was only one amino acid different between them), Myc-tag was added to the N-terminal of DN-u-calpain. These constructs were expressed in stable rat L8 myotubes and their effects on a variety of targets were examined. Down-regulation of u-calpain activity by the constructs was confirmed by the stabilization of fodrin (an index of calpain activity). Our data supported the contention that u-calpain contributes significantly to total protein degradation in myotubes. For instance, over-expressing DN-u-calpain reduced total protein degradation by 7.9% ($P<0.01$) at 24 hr time point and by 10.65% ($P<0.01$) following 24 hr of ponasterone A treatment and following 48 hr. Similarly, over-expression of antisense u-calpain and the 30K subunit reduced total protein degradation significantly following 24 hr of ponasterone A treatment ($P<0.05$). However, over-expression of the fused antisense (80K/30K) did not affect ($P>0.05$) total protein degradation. Based on the ability to regulate the activity of calpains, we demonstrated that desmin was a calpain substrate, but tropomyosin was not a substrate of calpain.

A second goal of this study was to evaluate the expression relationship among u- and m-calpains and the 30K subunit. The rationale for this study was that our earlier work indicated coordinated regulation of the calpain subunits and complicated activation phenomenon of u- and m-calpains. Taking advantages of our cell lines, we demonstrated for the first time that there was a coordinated expression relationship between m-calpain and the 30K subunit, and between u-calpain and the 30K subunit. And, we have evidence

for a similar coordinated expression between u- and m-calpains. A reduction in 30K subunit expression increased u- and m-calpain expression at both mRNA and protein levels. Over-expression of DN-u-calpain increased 30K expression at both mRNA and protein levels.

The third goal of this study was to develop a skeletal muscle-specific inducible expression system for later transgenic animal research. A skeletal muscle a-actin promoter was used to replace the cytomegalovirus immediate-early promoter (pCMV) in the ecdysone inducible expression system. In order to test the function of the modified expression, the LacZ gene was used as a reporter gene. A beta-galactosidase staining assay and high-sensitivity B-gal activity assay indicated that the modified skeletal muscle-specific expression system functioned in myotubes, but not in myoblasts. After 48 hr of induction, B-Gal activity was 15-fold higher in treated myotubes than in the control (untreated) myotubes. The next phase of the work will combine this method with our earlier work to establish roles of calpains in muscle *in vivo*.

Bibliography

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. (1990) Basic local alignment search tool. J. Mol. Biol. 215:403-10.

Arthur G.D., Booker T.S., and Belcastro A.N. (1999) Exercise promotes a subcellular redistribution of calcium-stimulated protease activity in striated muscle. Can. J. Physiol. Pharmacol. 77:42-7.

Arthur, J.S.C., Elce, J.S., Hegadorn, C., Williams, K., and Greer, P.A. (2000) Disruption of the murine calpain small subunit gene, Capn4: calpain is essential for embryonic development but not for cell growth and division. Mole. Cell. Bio. 20:4474-81.

Asante, E.A., Boswell, J.M., Burt, D.W., and Bulfield, G. (1994). Tissue specific expression of an α-skeletal actin-lacZ fusion gene during development in transgenic mice. Transgenic Research. 3, 59-66.

Atencio I.A., Ramachandra M., Shabram P., and Demers G.W. (2000) Calpain inhibitor 1 activates p53-dependent apoptosis in tumor cell lines. Cell Growth Differ. 11:247-53.

Bardsley R.G., Allcock S.M., Dawson J.M., Dumelow N.W., Higgins J.A., Lasslett Y.V., Lockley A.K., Parr T., and Buttery P.J. (1992) Effect of beta-agonists on expression of calpain and calpastatin activity in skeletal muscle. Biochimie. 74:267-73.

Barnes T.M., and Hodgkin J. (1996) The tra-3 sex determination gene of Caenorhabditis elegans encodes a member of the calpain regulatory protease family. EMBO J. 15:4477-84.

Barnoy S., Glaser T., and Kosower N.S. (1998) The calpain-calpastatin system and protein degradation in fusing myoblasts. Biochim. Biophys. Acta. 1402:52-60.

Bartus R.T., Hayward N.J., Elliott P.J., Sawyer S.D., Baker K.L., Dean R.L., Akiyama A., Straub J.A., Harbeson S.L., Li Z., and Powers, J. (1994) Calpain inhibitor AK295 protects neurons from focal brain ischemia. Effects of postocclusion intra-arterial administration. Stroke. 25:2265-70.

Blanchard H., Grochulski P., Li Y., Arthur J.S., Davies P.L., Elce J.S., and Cygler M. (1997) Structure of a calpain Ca(2+)-binding domain reveals a novel EF-hand and Ca(2+)-induced conformational changes. Nat. Struct. Biol. 4:532-8.

Capetanaki Y., and Milner D.J. (1998) Desmin cytoskeleton in muscle integrity and function. Subcell. Biochem. 31:463-95.

Dambadur, R., Sharma, M., Smith, T.P., and Bass, J.J. Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle. Genome Res. 1997 7:910-6.

Dayton, W.R., Goll, D.E., Stromer, M.H., Reville, W.J., Zeece, M.g., and Robson, R.M. (1975) Some properties of a Ca2+-activated protease that may be involvedin myofibrillar protein turnover. In *Cold Spring Harbor Conferences on Cell Proliferation*, vol. 2, *Proteases and Biological Control*, eds. Reich, E., Rifkin, D.B., and Shaw E., 551-7. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Dear, N., Matena, K., Vingron, M., and Boehm, T. (1997) A new subfamily of vertebrate calpains lacking a calmodulin-like domain: implications for calpain regulation and evolution. Genomics 45:175-84.

Delaney, S.J., Hayward, K.C., Barleben, F., Fischbach, K.F., and Gabor Miklos, G.L. (1991) Molecular cloning and analysis of small optic lobes, a structural brain gene of *Drosophila melanogaster*. Proc. Natl. Acade. Sci. USA. 88:7213-18.

Denison, S. H., Orejas, M., and Arst, H.N., Jr. (1995) Signaling of ambient pH in *Aspergillus* involves a cysteine protease. J. Biol. Chem. 270:28519-22.

Elamrani N., Brustis J.J., Dourdin N., Balcerzak D., Poussard S., Cottin P., and Ducastaing A. (1995) Desmin degradation and Ca(2+)-dependent proteolysis during myoblast fusion. Biol. Cell.85:177-83.

Elce J.S., Davies P.L., Hegadorn C., Maurice D.H., and Arthur J.S. (1997) The effects of truncations of the small subunit on m-calpain activity and heterodimer formation. Biochem. J. 326:31-8.

Elce, J.S., Hedadorn, C., and Arthur, J.S.C. (1997) Autolysis, Ca2+ requirement, and heterodimer stability in m-calpain. J. Biol. Chem. 272:11268-75.

Emori, Y., and Saigo, K. (1994) Calpain localization changes during early embryonic development of Drosophila. J. Bio. Chem. 269:25173-42.

Farah C.S., and Reinach F.C. 91995) The troponin complex and regulation of muscle contraction. FASEB J. 9:755-67.

Figueiredo-Pereira M.E., Banik N., and Wilk S. (1994) Comparison of the effect of calpain inhibitors on two extralysosomal proteinases: the multicatalytic proteinase complex and m-calpain. J. Neurochem. 62:1989-94.

Forsberg N.E., Ilian M.A, Ali-Bar A., Cheeke 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. 67:3313-21.

Gallagher, S., Winston, S.E., Fuller, S.A., and Hurrell, J.G.R. (2000) Immunoblotting and immunodetection. In *Current Protocols of Molecular Biology*, 10.8.1-10.8.21. eds. by

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. John Wiley & Sons, Inc.

Gao W.D., Atar D., Liu Y., Perez N.G., Murphy A.M., and Marban E. (1997) Role of troponin I proteolysis in the pathogenesis of stunned myocardium. Circ. Res. 80:393-9.

Goebel H.H., and Warlo I. (2000) Gene-related protein surplus myopathies. Mol. Genet. Metab. 71:267-75.

Goldberg, A.L. (1969a) Protein turnover in skeletal muscle. I. Protein catabolism during work-induced hypertrophy and growth induced with growth hormone. J. Biol. Chem. 244:3217-22.

Goldberg, A.L. (1969b) Protein turnover in skeletal muscle. II. Effects of denervation and cortisone on protein catabolism in skeletal muscles. J. Biol.Chem. 244:3223-9.

Goll D.E., Dayton W.R., Singh I., and Robson R.M. (1991) Studies of the alpha-actinin/actin interaction in the Z-disk by using calpain. J. Biol. Chem. 266:8501-10.

Goll D.E., Thompson V.F., Taylor R.G., and Christiansen J.A. (1992a) Role of the calpain system in muscle growth. Biochimie. 74:225-37.

Goll, K.E., thompson, V.F., and Taylor, R.G., and Zalewska, T. (1992b) Is calpain activity regulated by membranes and autolysis or by calcium and calpastatin? BioEssays 14:549-56.

Grobet, L., Martin L.J., Poncelet, D., Pirottin, D., Brouwers, B., Riquet, J., Schoeberlein, A., Dunner, s., Menissier, F., Massabanda, J., Fries, R., Hanset, R., and Georges, M. (1997) A deletion in the bovine myostatin gene causes the double-muscled phenotype in cattle. Nat. Genet. 17:71-4.

Groll M., Ditzel L., Lowe J., Stock D., Bochtler M., Bartunik H.D., and Huber R. (1997) Structure of 20S proteasome from yeast at 2.4 A resolution. Nature 386:463-71.

Hanada, K., Tamai, M., Morinoto, S., Yamagishi, M., Ohmura, S., Sawada, J., and Tanaka, I. (1978) Studies on thiol ptotease inhibitors. PartI. Isolotion and characterisation of E64, a new thiol protease inhibitor. Agric. Biol. Chem. 42:523-8.

Harriman J.F., Waters-Williams S., Chu D.L., Powers J.C., and Schnellmann R.G. (2000) Efficacy of novel calpain inhibitors in preventing renal cell death. J. Pharmacol. Exp. Ther. 294:1083-7.

Hata A., Ohno S., and Suzuki K. (1992) Transcriptional activation of the gene for the large subunit of human m-calpain by 12-o-tetradecanoyl-phorbol-13-acetate. FEBS Lett. 304:241-4.

Hosfield, C.M., Elce, J.S., Davies, P.L., and Jia, Z. (1999) Crystal structure of calpain reveals the structural basis for Ca(2+)-dependent protease activity and a novel mode of enzyme activation. EMBO J. 18:6880-9.

Huang J., and Forsberg N.E. (1998) Role of calpain in skeletal-muscle protein degradation. Proc. Natl. Acad. Sci. USA. 95:12100-5.

Ilian M.A., and Forsberg N.E. (1992) Gene expression of calpains and their specific endogenous inhibitor, calpastatin, in skeletal muscle of fed and fasted rabbits. Biochem J. 287:163-71.

Jiang Q., and Stys P.K. (2000) Calpain inhibitors confer biochemical, but not electrophysiological, protection against anoxia in rat optic nerves. J. Neurochem. 74:2101-7.

Johnson, G.V., and Guttmann R.P. (1997) Calpains: intact and active? Bioessays 19:1011-8.

Kretchmar D.H., Hathaway M.R., Epley R.J., and Dayton W.R. (1989) In vivo effect of a beta-adrenergic agonist on activity of calcium-dependent proteinases, their specific inhibitor, and cathepsins B and H in skeletal muscle. Arch. Biochem. Biophys. 275:228-35.

Kusakawa G., Saito T., Onuki R., Ishiguro K., Kishimoto T., and Hisanaga S. (2000) Calpain-dependent proteolytic cleavage of the p35 cyclin-dependent kinase 5 activator to p25. J. Biol. Chem. 275:17166-72.

Lankiewicz S., Marc Luetjens C., Truc Bui N., Krohn A.J., Poppe M., Cole G.M., Saido T.C., and Prehn J.H. (2000) Activation of calpain I converts excitotoxic neuron death into a caspase-independent cell death. J. Biol. Chem. 275:17064-71.

Lee M.S., Kwon Y.T., Li M., Peng J., Friedlander R.M., and Tsai L.H. (2000) Neurotoxicity induces cleavage of p35 to p25 by calpain. Nature 405:360-4.

Lee, H.J., Sorimachi, H., Jeong, S.Y., Ishiura, S., and Suzuki, K. (1998) Molecular cloning and characterizatin of a novel tissue-specific calpain predominantly expressed in the digestive tract. Bio. Chem. 379:175-83.

Li Z., Mericskay M., Agbulut O., Butler-Browne G., Carlsson L., Thornell L.E., Babinet C., and Paulin D. (1997) Desmin is essential for the tensile strength and integrity of myofibrils but not for myogenic commitment, differentiation, and fusion of skeletal muscle. J. Cell. Biol. 139:129-44.

Lin G.D., Chattopadhyay D., Maki M., Wang 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. Struct. Biol. 4:539-47.

Lin G.D., Chattopadhyay D., Maki M., Wang 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. Struct. Biol. 4:539-47.

Liu K., Li L., and Cohen S.N. (2000) Antisense RNA-mediated deficiency of the calpain protease, nCL-4, in NIH3T3 cells is associated with neoplastic transformation and tumorigenesis. J. Biol. Chem. 275:31093-8.

Low, R.B. and Goldberg, A.L. (1973) Non-uniform rates of turnover of myofibrillar proteins in rat diaphragm. J. Cell Biol. 56: 590-5.

Ma, H., Fukiage, C., Azuma, M., and Shearer, T.R. (1998) Molecular cloning and expression of calpain Lp82 from rat lens: A splice variant of muscle p94 mRnA with insert refions deleted. Investigat. Ophthalmol. and Vis. Sci. 39:454-61.

Ma, H., Hata, I., Fukiage, C., Azuma, M., and Shearer, T.R. (2000) Lp85 calpain is an enzymatically active rodent-specific isozyme of lens Lp82. Curr. Eye Res. 20:183-9.

Maruyama K., Usami M., Kametani F., Tomita T., Iwatsubo T., Saido T.C., Mori H., and Ishiura S. (2000) Molecular interactions between presenilin and calpain: inhibition of m-calpain protease activity by presenilin-1, 2 and cleavage of presenilin-1 by m-, mu-calpain. Int. J. Mol. Med. 5:269-73.

McPherron, A.C., and Lee, S.J. (1997a) Double muscling in cattle due to mutations in the myostatin gene. Proc. Natl. Acad. Sci. USA. 94:2457-61.

McPherron, A.C., and Lee, S.J. (1997b) Regulation of skeletal muscle mass in mice by a new TGF-beta super family member. Nature 387:83-90.

Melloni E., Averna M., Salamino F., Sparatore B., Minafra R., and Pontremoli S. (2000) Acyl-CoA-binding protein is a potent m-calpain activator. J. Biol. Chem. 275:82-6.

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. 249:583-8.

Milner D.J., Mavroidis M., Weisleder N., and Capetanaki Y. (2000) Desmin cytoskeleton linked to muscle mitochondrial distribution and respiratory function. J. Cell. Biol. 150:1283-98.

Nath R., Davis M., Probert A.W., Kupina N.C., Ren X., Schielke G.P., and Wang K.K. (2000) Processing of cdk5 activator p35 to its truncated form (p25) by calpain in acutely injured neuronal cells. Biochem. Biophys. Res. Commun. 274:16-21.

Nelson W.J., and Traub P. (1983) Proteolysis of vimentin and desmin by the Ca2+-activated proteinase specific for these intermediate filament proteins. Mol. Cell. Biol. 3:1146-56.

Nishiura, I., Tanaka, K., Yamato, S., and Murachi, T. (1978) The occurrence of an inhibitor of Ca2+-dependent neutral protease in rat liver. J. Biochem. 84:1657-9.

No, D., Yao, T.P., and Evans, R.M. (1996). Ecdysone-inducible gene expression in mammalian cells and transgenic mice. Proc. Natl. Acad. Sci. USA. 93, 3346-3351.

Ono, Y., Sorimachi, H. and Suzuki, K. (1999) The calpain superfamily. In *Calpain--Pharmacology and Toxicology of Calcium-Dependent Protease.* eds: Wang K.K.W. and Yuen, P.-W. 1--23. Taylor & Francis, Philadelphia, PA.

Parr T., Bardsley R.G., Gilmour R.S., and Buttery P.J. (1992) Changes in calpain and calpastatin mRNA induced by beta-adrenergic stimulation of bovine skeletal muscle. Eur. J. Biochem. 208:333-9.

Patrick, G.N., Zukerberg, H., Nikolic, M., de la Monte, S., Dikkes, P., and Tsai, L.H., (1999) Neurotoxicity induces cleavage of p35 to p25 by calpain. Nature 402:625-22.

Rami A., Agarwal R., Botez G., and Winckler J. (2000) mu-Calpain activation, DNA fragmentation, and synergistic effects of caspase and calpain inhibitors in protecting hippocampal neurons from ischemic damage. Brain Res. 866:299-312.

Ray S.K., Fidan M., Nowak M.W., Wilford G.G., Hogan E.L., and Banik N.L. (2000) Oxidative stress and Ca2+ influx upregulate calpain and induce apoptosis in PC12 cells. Brain Res. 852:326-34.

Ray S.K., Matzelle D.C., Wilford G.G., Hogan E.L., and Banik N.L. (2000) E-64-d prevents both calpain upregulation and apoptosis in the lesion and penumbra following spinal cord injury in rats. Brain Res. 867:80-9.

Reid W.D., Huang J., Bryson S., Walker D.C., and Belcastro A.N. (1994) Diaphragm injury and myofibrillar structure induced by resistive loading. J. Appl. Physiol. 76:176-84.

Richard I., Broux O., Allamand V., Fougerousse F., Chiannilkulchai N., Bourg N., Brenguier L., Devaud C., Pasturaud P., Roudaut C., et al (1995) Mutations in the proteolytic enzyme calpain 3 cause limb-girdle muscular dystrophy type-2A. Cell 81:27-40.

Saatman K.E., Murai H., Bartus R.T., Smith D.H., Hayward N.J., Perri B.R., and McIntosh T.K (1996) Calpain inhibitor AK295 attenuates motor and cognitive deficits following experimental brain injury in the rat. Proc. Natl. Acad. Sci. USA. 93:3428-33.

Saatman K.E., Zhang C., Bartus R.T., and McIntosh T.K. (2000) Behavioral efficacy of post-traumatic calpain inhibition is not accompanied by reduced spectrin proteolysis, cortical lesion, or apoptosis. J. Cereb. Blood Flow Metab. 20:66-73.

Saez E., Nelson M.C., Eshelman B., Banayo E., Koder A., Cho G.J., and Evans R.M. (2000) Identification of ligands and coligands for the ecdysone-regulated gene switch. Proc. Natl. Acad. Sci. USA. 97:14512-7.

Saido T.C., Nagao S., Shiramine M., Tsukaguchi M., Yoshizawa T., Sorimachi H., Ito H., Tsuchiya T., Kawashima S., and Suzuki K. (1994) Distinct kinetics of subunit autolysis in mammalian m-calpain activation. FEBS Lett. 346:263-7.

Saido, T.C., Sorimachi, H., and Suzuki, K. (1994) Calpain: new perspectives in molecular diversity and physiological-pathological involvement. FASEB J. 8:814-22.

Samaha F.J., and Quinlan J.G. Dystrophinopathies: clarification and complication. J. Child. Neurol. 11:13-20.

Sasaki T., Kikuchi T., Yumoto N., Yoshimura N., and Murachi T. (1984) Comparative specificity and kinetic studies on porcine calpain I and calpain II with naturally occurring peptides and synthetic fluorogenic substrates. J. Biol. Chem. 259:12489-94.

Sato, N., Murata, K., Watanabe, K., Hayami, T., Kariya, Y., Sakaguchi, M., Kimura, S., Nonaka, M., and Kimura, A. (1988) Growth-promoting activity of tuna growth hormone and expression of tuna growth hormone cDNA in *Escherichia coli.* Biotech. Appli. Biochem. 10:385-93.

Schumacher P.A., Eubanks J.H., and Fehlings M.G. (1999) Increased calpain I-mediated proteolysis, and preferential loss of dephosphorylated NF200, following traumatic spinal cord injury. Neuroscience. 91:733-44.

Schumacher P.A., Siman R.G., and Fehlings M.G. (2000) Pretreatment with calpain inhibitor CEP-4143 inhibits calpain I activation and cytoskeletal degradation, improves neurological function, and enhances axonal survival after traumatic spinal cord injury. J. Neurochem. 74:1646-55.

Shields D.C., Schaecher K.E., Hogan E.L., and Banik N.L. (2000) Calpain activity and expression increased in activated glial and inflammatory cells in penumbra of spinal cord injury lesion. J. Neurosci. Res. 61:146-50.

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. 271:26690-7.

Sorimachi, H., Ishiura, S., and Suzuki, K. (1997) Structure and physiological function of calpains. Biochem. J. 328:721-32.

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 Ca(2+)-binding domain. J. Biol. Chem. 268:19476-82.

Sormachi, H., Kmajoh-Ohmi, L., Emori, Y., Kawasaki, H., Ohno, S., Minami, Y., and Suzuki, K. (1989) Molecular cloning of a novel mammalian calcium-dependent protease distinct form both m- and u--types. J. Biol. Chem. 264:20106-11.

Spencer M.J., and Tidball J.G. (1992) Calpain concentration is elevated although net calcium-dependent proteolysis is suppressed in dystrophin-deficient muscle. Exp. Cell. Res. 203:107-14.

Strobl S., Fernandez-Catalan C., Braun M., Huber R., Masumoto H., Nakagawa K., Irie A., Sorimachi H., Bourenkow G., Bartunik H., Suzuki K., and Bode W. (2000) The crystal structure of calcium-free human m-calpain suggests an electrostatic switch mechanism for activation by calcium. Proc. Natl. Acad. Sci. USA. 97:588-92.

Sugawara M., Kato K., Komatsu M., Wada C., Kawamura K., Shindo P.S., Yoshioka P.N., Tanaka K., Watanabe S., and Toyoshima I. (2000) A novel de novo mutation in the desmin gene causes desmin myopathy with toxic aggregates. Neurology. 55:986-90.

Sultan, K.R., Dittrich, B.T., and Pette, D. (2000) Calpain activity in fast, slow, transforming, and regenerating skeletal muscles of rat. Am. J. Physiol. Cell Physiol. 279:C639-47.

Tagawa K., Taya C., Hayashi Y., Nakagawa M., Ono Y., Fukuda R., Karasuyama H., Toyama-Sorimachi N., Katsui Y., Hata S., Ishiura S., Nonaka I., Seyama Y., Arahata K., Yonekawa H., Sorimachi H., and Suzuki K. (2000) Myopathy phenotype of transgenic mice expressing active site-mutated inactive p94 skeletal muscle-specific calpain, the gene product responsible for limb girdle muscular dystrophy type 2A. Hum. Mole. Genet. 9:1393-402.

Thompson, V.F., Lawson, K., and Goll, D.E. (2000) Effect of u-calpain on m-calpain. Biochem. Biophys. Res. Commun. 267:495-9.

Tompa P., Baki A., Schad E., and Friedrich P. (1996) The calpain cascade. Mu-calpain activates m-calpain. J. Biol. Chem. 271:33161-4.

Touyarot K., Poussard S., Verret C., Aragon B., Cottin P., Nogues X., and Micheau J. (2000) Calpain-PKC inter-relations in mouse hippocampus: a biochemical approach. Neurochem. Res. 25:781-90.

Uehara Y., Mori C., Noda T., Shiota K., and Kitamura N. (2000) Rescue of embryonic lethality in hepatocyte growth factor/scatter factor knockout mice. Genesis. 27:99-103.

Vasilatos-Younken, R., Zhou, Y., Wang, X., McMurtry J.P., Rosebrough, R.W., Decuypere, E., Buys, N., Darras, V.M., Van der Geyten, S., and Tomas, F. (2000) Altered chicken thyroid hormone metabolism with chronic GH enhancement *in vivo*: consenquences for skeletal muscle growth. J. Endocri. 166:609-20.

Wang K.K., Nath R., Posner A., Raser K.J., Buroker-Kilgore M., Hajimohammadreza I., Probert A. W., Marcoux 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. (1996) An alpha-mercaptoacrylic acid derivative is a selective nonpeptide cell-permeable calpain inhibitor and is neuroprotective. Proc. Natl. Acad. Sci. USA. 93:6687-92.

Warren R.H. (1997) TGF-alpha-induced breakdown of stress fibers and degradation of tropomyosin in NRK cells is blocked by a proteasome inhibitor. Exp. Cell. Res. 236:294-303.

Waterson, R., Martin, C., Craxton, M., Huynh, C., Coulson, A., Hillier, L., Durbin, R., Green, P., Shown-keen, R., Halloran, N., Metzstein, M., Hawkins, T., Wilson, r., Berks, M., Du, Z., thomas, K., ThierryMieg, J., and Sulston, J. (1992) A survey of expressed genes in *Caenorhabditis elegans*. Nature Genetics 1:114-23.

Waxman, L., and Krebs, E.G. (1978) Identification of two protenase inhibitors from bovine cardiac muscle. J. Biol. Chem. 253:5888-91.

Wilson, R., Alinscough, R. et al. (1994) 2.2 Mb of contiguous nucleotide sequence from chromsome III of *C. elegans*. Nature 368:32-8.

Yee, S.P., and Rigby, P.W. (1993) The regulation of myogenin gene expression during the embryonic development of the mouse. Genes Dev. 7:1277-89.

Yoshikawa Y., Mukai H., Hino F., Asada K., and Kato I. (2000) Isolation of two novel genes, down-regulated in gastric cancer. Jpn. J. Cancer Res. 91:459-63.

Yoshimura, N., Kikuchi, T., Sasaki, T., Kitahara, A., Hatanaka, M., and Murachi, T. (1983) Two distinct Ca^{2+}proteases (calpain I and calpain II) purified concurrently by the same method from rat kidney. J. Biol. Chem. 258:8883-9.

Yoshizawa T., Sorimachi H., Tomioka S., Ishiura S., and Suzuki K. (1995) Calpain dissociates into subunits in the presence of calcium ions. Biochem. Biophys. Res. Commun. 208:376-83.

Yoshizawa, T., Sorimachi, H., tomioka, S., Ishiura, S., and Suzuki, K. (1995) A catalytic subuint of calpain prossesses full proteolytic activity. FEBS Letters 358:101-3.

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 253:192-3.

Zhang J., Patel J.M., and Block E.R. (1998) Hypoxia-specific upregulation of calpain activity and gene expression in pulmonary artery endothelial cells. Am. J. Physiol. 275:L461-8.

Zhang W., and Mellgren R.L. (1996) Calpain Subunits Remain Associated during Catalysis. Biochem. Biophys. Res. Commun. 227:890-6.

Zhu D.M., and Uckun F.M. (2000) Calpain inhibitor II induces caspase-dependent apoptosis in human acute lymphoblastic leukemia and non-Hodgkin's lymphoma cells as well as some solid tumor cells. Clin. Cancer Res. 6:2456-63.

Zimmerman U.J., Boring L., Pak J.H., Mukerjee N., and Wang K.K. (2000) The calpain small subunit gene is essential: its inactivation results in embryonic lethality. IUBMB Life. 50:63-8.

Appendix

Appendix

Mixture-Screening Method for Identifying Bacterial Colonies Containing the Recombinant Plasmid DNA

A challenge encountered by beginning and seasoned molecular biologists is sub-cloning of a DNA fragment into a plasmid vector. Ligations are often challenging projects and various strategies haven been employed to enhance ligation efficiency (e.g., cohesive end ligation, choosing appropriate ratio of inserts to vectors; 1). However, scientists are often plagued with the problem of low ligation efficiency and, in this case, often expect a tremendous amount of labor in screening of bacterial colonies for one that contains a successfully ligated plasmid. Screening large-scale colonies could be done with polymerase chain reaction (PCR) or by hybridization techinques (1,2); however, these approaches are also time consuming and expensive. The cracking PCR might be helpful (6). But its success depends on the success of the first step cracking and the success of following PCR. In some instances, it is very difficult to achieve success. TA cloning is another alternation to enhance subcloning efficiency, but this requires recovering the insert from the TA cloning vector as an intermediate step. This, too, is a laborious step.

In our experiment, we needed to subclone the rat micro-calpain large subunit (u-CL, 2,200bp) (5) from pUC18 (in EcoRI site) into pIND (5.0kb, Invitrogen, Carlsbad, CA) between BamHI and EcorRI sites. We created extra bases flanking both restriction sites in the polymerase chain reaction (PCR) product of u-CL. After PCR, we directly used restriction endonucleases BamHI (New England Biolabs, Beverly, MA), and EcoRI (Stratagene, La Jolla, CA) to double-digest the PCR product in Buffer H (Promaga, Madison, WI). Then, the digested product was purified via agarose gel electrophoresis (1%) and the correct bands were recovered for purifying the digested PCR product in

Glassmilk (Geneclean II Kit) (Bio101 Inc., Vista, CA). The pIND was also double-digested with BamHI and EcoRI, and the product was purified as the digested PCR product. According to the concentration of digested PCR product and pIND, the ligation was processed in a molar ratio of insert to vector of 1:1 and 3:1 with a Rapid DNA Ligation Kit (Boehringer Mannheim GmbH, Germany). After a 5-minute ligation, 10ul of the ligation product was used to transform self-made *E. coli* DH5a competent cells (100ul). After transformation and one-hour incubation in LB medium, 80 ul of the culture medium from each ligation were spread on an LB plate containing 50 ug/ml ampicillin. The plates were incubated overnight at 37°C.

Individual colonies were randomly picked up from different ligation plates and inoculated into 2 ml LB medium (containing ampicillin). After overnight incubation (37°C, 250 RPM shaking), an alkaline lysis procedure was used to make minipreps for each colony (1). A 5ul aliquot of plasmid DNA mimiprep was digested by EcoRI and electrophoresed on a 1% agarose gel. After screening 48 colonies (four groups, 12 colonies per group), we finally recovered one colony containing the recombinant plasmid DNA (Figure A.1). Double digestion of the miniprep further verified the insert size and the correct orientation of the ligation.

Normal Procedure

After ligation, one usually cultures ten or twenty individual transformed colonies (e.g., 100 colonies) overnight and makes minipreps. Then, restriction endonucleases are used to digest the minipreps to verify the ligation. If no ligation is verified, another ligation reaction will be performed. However, lack of detecting a successful ligation does not conclusively mean that there is no ligation product from these 100 colonies. The desired ligation product may simply exist at very low abundance, (e.g., 1 out of 100). The key challenge is how to rapidly identify bacterial colonies that contain the low abundance recombinant plasmid DNA. The rapid disruption of colonies to test for recombinant plasmid DNA can be an alternative (3). But this method requires that the colonies are

grown to large size (2-3 mm). Sometimes this requirement cannot be met. Even the method for rapid preparation of plasmid DNA (4) does not help to resolve the problem of identifying the successful ligation product in low abundance. We developed a mixture-screening method to minimize the labor required to screen bacterial colonies resulting from low-efficient ligation. The method used to accomplish this is outlined in Table A.1.

Mixture-Screening Procedure

In order to overcome the problem of low ligation efficiency, we developed the mixture screening method (Table A.1). Again, EcoRI digestion of plasmid DNA was used as a diagnostic strategy for identification of successful ligation. Clearly, in mixture 12 of the plasmid DNA minipreps, there was at least one colony that contained the recombinant plasmid DNA (Figure A.2). The individual colonies in mixture 12 were amplified and their plasmid DNA was double-digested by EcoRI and BamHI. Clearly, the colony 9 contained the recombinant plasmid DNA (Figure A.3). Because Figure A.2 is not completely clear, we have to be cautious in claiming that there were more colonies containing the recombinant plasmid DNA. But according the ligation efficiency of the individual screening (1 ligation out of 48 colonies), from screening 120 colonies we should get at least two mixtures which contained the colony which had recombinant plasmid DNA. Double digestion was performed for the other eleven mixtures. Obviously, in the mixtures 1 and 8 there were at least one colony containing the recombinant plasmid DNA in these two groups, respectively (Figure A.4).

The mixture screening method is very efficient in increasing the opportunity of recovering bacterial colonies containing recombinant plasmid DNA, especially in the case of low ligation efficiency. The number of mixed colonies in each mixture and the total number of screened mixtures can be changed according to the estimation of ligation efficiency. Even though one recombinant plasmid will only produce one tenth of the total plasmid DNA, the insert still can produce sufficient signals for distinguishing the miniprep mixtures. If the insert has compatible ends, the orientation of the ligation is

another major concern after ligation. Double digestion of the mixture minipreps can be carefully designed to check orientation of the ligation. So, double digestion can be performed to check not only the possible ligation in the mixtures, but also the orientation of the ligation.

References

1. Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl. (1994) Current Protocols in Molecular Biology. John Wiley & Sons, Inc.

2. Grunstein, M. and Hogness D. (1975) Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. USA. 72:3961-3.

3. Barnes, W.M. (1977) Plasmid detection and sizing in single colony lysates. Science 195:393-4.

4. Li, B., Plicher K.Y., Wyman T.E., and Machida C.A. (1997) Rapid preparation and identification of insert-containing recombinant plasmid DNA. Biotechniques 23:603-8.

5. Sorimachi H., Amano S., Ishiura S., and Suzuki K. (1996) Primary sequences of rat u-calpain large and small subunits are respectively, moderately and highly similar to those of human. Biochim. Biophys. Acta 1039:37-41.

6. Campbell T.N., and Choy R.Y.M. (2001) Large-scale colony screening and insert orientation determination using PCR. BioTechniques 30:32-4.

Table A.1. Procedure of Mixture Screening

Step 1. Ten individual colonies are separately picked up by sterile toothpicks or pipet tips and dispensed into ten 500ul-Eppendorf tubes, each containing 50ul LB medium. After dispensing, each tip is used to spread a small area on one LB plate (containing Amp or other antibiotics). The surface 100mm-plate is divided into 50 parts. The plate is incubated at 37°C overnight.

Step 2. Collect the medium from the ten 500ul-Eppendorf tubes and inoculate 2 ml LB medium (containing antibiotics) in a 15-ml tube. Incubate the 15-ml tube overnight at 37°C with shaking at 250 RPM.

Step 3. Go through the same procedure for the other eleven groups (10 colonies/group). The plates and the tubes are marked in order.

Step 4. Make plasmid DNA minipreps (1) and digest the minipreps to identify the mixture which contains the recombinant plasmid DNA.

Step 5. Ten colonies representing the original ten colonies in their respective plates are picked-up to separately inoculate into 2 ml LB medium containing antibiotics in a 15 ml tube. The ten tubes are incubated overnight at 37°C with shaking at 250 RPM.

Step 6. Make minipreps for the ten overnight cultures and digest the minipreps to find out the miniprep/colony that contains the recombinant plasmid DNA.

Note: In 20 minipreps, 100 colonies are screened.

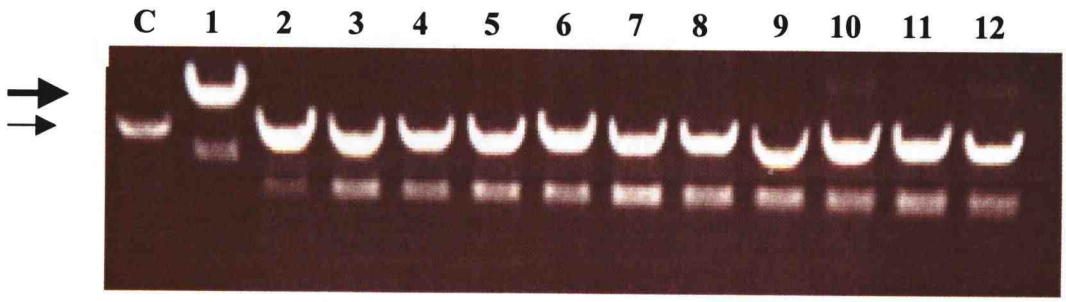


Figure A.1. Individual-colony screening for the recombinant plasmid DNA. Lane C represents the vector positive control. In Lane 1 the large band size is 7.2 Kb (insert 2.2 Kb plus vector 5.0 Kb). The large arrow points to the recombinant plasmid DNA. The small arrow points to the vector-only band.

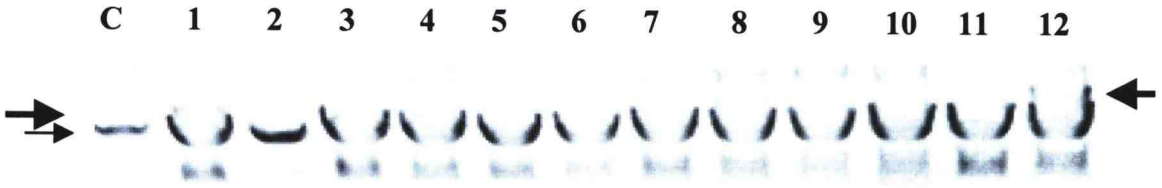


Figure A.2. Electrophoresis for mixture-screening. The recombinant plasmid DNA band position is indicated by large arrow (vector plus insert 7.2 kb) and the vector alone is indicated by a small arrow (Lane C). Clearly, in Lane 12 there was a recombinant plasmid DNA band. In Lanes 1 and 8 the recombinant bands were not so clear.

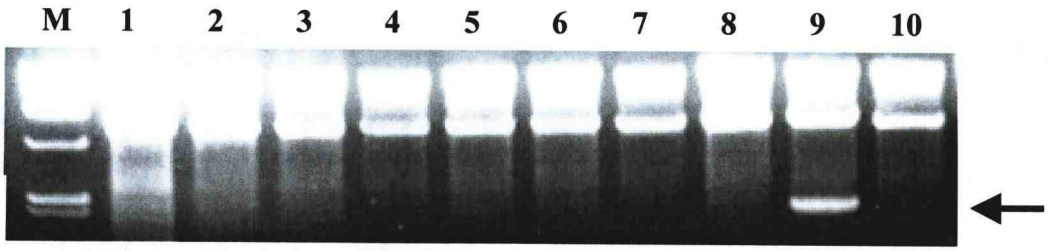


Figure A.3. Confirmation of the recombinant plasmid DNA in Mixture 12. Individual colonies in Mixture 12 were amplified and their plasmid DNA was double-digested by BamHI and EcoRI. Clearly, Colony 9 contained the recombinant plasmid DNA. The insert 2.2 kb was indicated by an arrow.

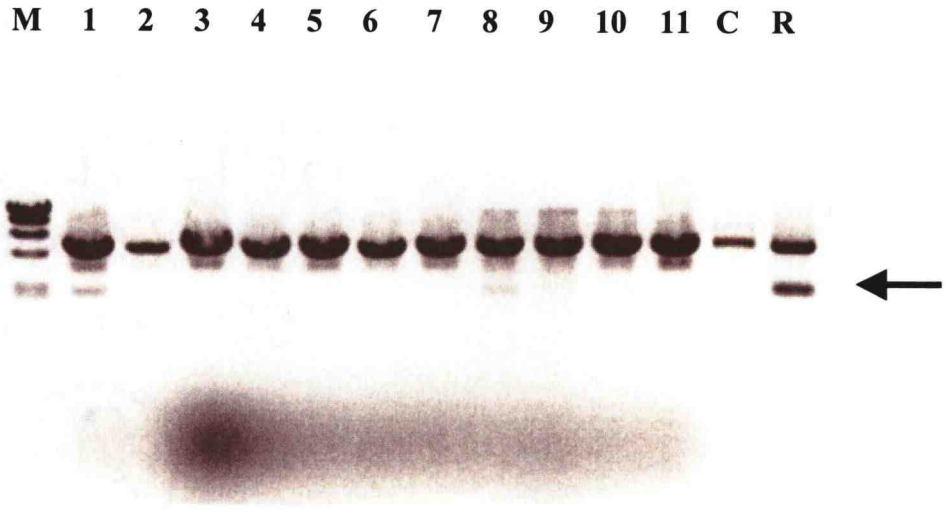


Figure A.4. Verifying other mixtures which contain recombinant plasmid DNA by double-digestion. Clearly, there are at least another two colonies containing the insert (Mixtures 1 and 8). Lane M was marker λ DNA/HinDIII digestion standard and Lane C was vector alone. The Lane R was a positive control colony 9 from Mixture 12 in Figure A.3.