DIII domain of calpain 10 and Cpl: towards an understanding of calpain 10 function

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FINAL APPROVAL OF DISSERTATION
Doctor of Philosophy in Medical Sciences

DIII Domain of Calpain 10 and Cpl Towards an Understanding of Calpain 10 Function

Submitted by

Xinhua Huang

In partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Medical Sciences

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Date of Defense: August 27, 2003
DIII Domain Of Calpain 10 And Cpl: Towards An Understanding Of Calpain 10 Function

Xinhua Huang

Medical College of Ohio

2003
DEDICATION

I would like to dedicate this dissertation to my family and my senior high school friend Peng Wei.
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INTRODUCTION

Calpains are intracellular, cysteine proteases found in mammalian cells and in some lower organisms (Delaney et al., 1991; Sorimachi et al., 1997; Huang and Wang, 2001). There is evidence that they are important mediators of cell adhesion and motility in animal cells (Shiraha et al., 1999). While there is evidence that the single nucleotide polymorphisms in the non-coding region of calpain-10 gene are associated with type 2 diabetes (Horikawa et al., 2000), few molecular and biochemical studies have been done on calpain-10. Because the cellular slime mold Dictyostelium discoideum is a genetically tractable model for cellular studies (Kessin, 2001), in this organism we have investigated a calpain-10-like protein which may provide useful clues towards an understanding of the function of calpain-10. Contig 13130 (Sanger Institute Dictyostelium sequencing project) was identified as a DNA sequence containing 3-exons that encodes a calpain-10-like protein. Using a custom peptide antibody to assay for the presence of this putative protein, we identified Dictyostelium calpain-like protein (Cpl) and purified it to near homogeneity. Dictyostelium calpain-like protein (Cpl) is a 72,278 Da cytosolic protein. Weak caseinolytic activity inhibitable by cysteine protease inhibitors was co-purified with Cpl immunoreactivity, and purified Cpl appeared to undergo autoproteolysis when transferred to inhibitor-free buffer. The major cleavage after Pro189 generated a 51,291 Da Cpl fragment. The Cpl domain structure resembles mammalian calpain-10 with an N terminal catalytic domain followed by tandem calpain D-III domains. The physiological function of the Cpl awaits further study. Calpain 10 was the first positional cloned gene associated with type 2 diabetes (Horikawa et al., 2000). In general, the physiological
roles of calpain-10 was even less known when compared with calpain 3 to disease limb girdle muscular dystrophy type 2A (LGMD2A). Single nucleotides polymorphism (SNP) investigation in different ethnic groups and geographies indicated that it is an influential factor in the polygenic disease type 2 diabetes (Cox, 2002). Yeast two-hybrid screening of a human liver library using Gal4BD-calpain DIIIX2 as bait revealed several positive clones, including flotillin-1. The positive cDNAs specifically interacted with calpain-10 DIIIX2, when tested with baits comprising the DIII domains from different calpains. Confirmation of protein-protein interaction by pulldown assay and cellular function studies of these positive clones await further work.
Mammalian calpains comprise a large family with at least 12 members (Huang and Wang, 2001), having sequence homology to the large subunits of the two classical calpains: calpain 1 (µ-calpain) and calpain 2 (m-calpain). In addition, the classical calpains contain a regulatory small subunit encoded by the Capn4 gene. There are several calpain-like proteins in non-mammalian organisms with homology to the calpain catalytic domain (Sorimachi and Suzuki, 2001). Proteins homologous to mammalian calpains were found in lower organisms such as fungi (Denison et al., 1995), Caenorhabditis elegans (Barnes and Hodgkin, 1996; Sorimachi et al., 1997) and Drosophila melanogaster (Delaney et al., 1991; Emori and Siago, 1994; Theopold et al. 1995).

Calpains 1 and 2 are intracellular, non-lysosomal proteases involved in apoptosis (Wolf et al., 1999), neural injury (Saatman et al., 1996,2000; Ray et al., 1999), neuron necrotic death (Wang, 2000), cell cycle (Mellgren et al., 1996), immune cell function (Noguchi et al., 1996; Ruiz-Vela et al., 1999; Lokuta et al., 2003) and platelet aggregation (Croce et al., 1999). Recently, calpains have been proposed to function in insulin secretion from mouse pancreatic islets (Sreenan et al., 2001). Calpains 1 and 2 require calcium ion for their activity (Khorchid and Ikura, 2002; Moldoveanu et al., 2002). There are EF-hand containing calcium binding motifs in domains IV and VI of the classical calpains (Sorimachi et al., 1989) and the activation of calpain 2 involved cooperation of several calcium binding sites to form an active enzyme (Moldoveanu et al., 2002). Whether non-classical calpains require calcium for their activation is still an open question.
Calpain family members may have specific functions in different tissues or organisms. The *Tra-3* gene, which corresponds to calpain 5 in mammalian cells, is part of the sex determination pathway in *C. elegans* (Barnes and Hodgkin, 1996). Calpain 3, also named p94, is mainly expressed in skeletal muscle tissue (Federici et al., 1999; Herasse et al., 1999), and loss of its proteolytic activity by genetic mutation is the cause of human limb girdle muscular dystrophy type 2A (LGMD2A) (Richard et al., 1995; Fougerousse et al., 1998; Ono et al., 1998). Calpain 10 was discovered to be associated with type 2 diabetes in Mexican-American and some European populations (Horikawa et al., 2000; Cox, 2002). Unlike calpain 1 and calpain 2 proteins, which are composed of two heterogeneous subunits, the other calpain proteins appear to consist of only one type of peptide homologous to the large catalytic subunit of calpains 1 and 2 (Huang and Wang, 2001). While calpain 3 protein is believed to be a homodimer (Kinbara et al., 1998), based on properties of the purified protein, the composition of the other newly discovered calpain family members remains unclear.

**Classical Calpains: Calpain 1 and Calpain 2**

Calpains 1 and 2 are Ca$^{2+}$-dependent cysteine endopeptidases (Wang and Yuen, 1999). Domain DI of their large subunit associates with the regulatory small subunit (Sorimachi and Suzuki, 2001. Fig. 1). This interaction is believed to influence calcium binding essential for formation of an active catalytic conformation in the large subunit (Hosfield et al., 1999). However, other investigators report that the small subunit is not necessary for calpain 1 or 2 protease activity (Yoshizawa et al., 1995). Domain DII of the large subunit is a catalytic domain with a conserved Cys, His, Asn catalytic triad essential
for enzymatic activity (Arthur et al., 1995). In multicellular organisms, Domain III can only been found in calpain family members so far (Sorimachi and Suzuki, 2001). To date, the function of the DIII domain remains unknown (Huang and Wang, 2001; Sorimachi and Suzuki, 2001; ). We propose that the DIII domains, especially in calpain-10, are responsible for interaction with other protein(s), either targeting the calpains to substrates or to protein complexes that contain a calpain substrate. Domain IV is highly similar to domain VI in calpain small subunit, both containing 5 EF-hand motifs. The 5th EF-hand motif (EF-5s) of domain IV and VI interact with each other to form the heterodimeric classic calpains (Wang and Yuen, 1999; Sorimachi and Suzuki, 2001).

Fig. 1. Schematic Domain Structure of Calpain 1

Calpastatin is a specific endogenous inhibitory peptide for calpain 1 and calpain 2, binding through the calcium-binding domain VI of calpain (Todd et al., 2003). Both calpain 1/calpain 2 and calpastatin are widely distributed in various animal tissues and cells (Blomgren et al., 1989; Thompson and Goll, 2000). Recent study of calpain 2 argues
for a multi-site cooperative interaction essential to the formation of a papain-like catalytic domain upon calcium binding (Reverter et al., 2001; Jia et al., 2002). In general, a low (µM) Ca\(^{2+}\) concentration is required for calpain 1 activation, and high (mM) Ca\(^{2+}\) concentration is required for calpain 2 activation \textit{in vitro} (Wang and Yuen, 1999). Although some literature suggested that calpain 1 needs \(\sim10-50\) µM Ca\(^{2+}\) for its activation and calpain 2 (Fig. 2) needs \(\sim300-500\) µM Ca\(^{2+}\) for its activation (Hosfield et al., 1999), both calcium concentrations for calpains activation are still much higher than the physiological intracellular Ca\(^{2+}\) concentration. Part of \textit{in vivo} calpain activity may be accounted for by localized and transient elevation of the free intracellular calcium concentration to 200-300 µM (Spira et al., 2001). It has been reported that interaction with phospholipid membranes could reduce the calcium requirement for activation (Chakrabarti et al., 1996; Reverter et al., 2001), and one early study reported that the polyphosphoinositide PIP\(_2\) could specifically promote calpain activation in the presence of 0.1 nM to 1 nM Ca\(^{2+}\) (Saido et al., 1992). A proposed \textit{in vitro} model suggested that the oscillation frequency of intracellular calcium may control the physiological activation of calpain (Tompa et al., 2001). The mechanism of calpain activation \textit{in vivo} remains to be elucidated.

After the protease core (domain I-II) of calpain 1 was expressed in \textit{Escherichia coli} and purified (Moldoveanu et al., 2002), it was impressively found containing only 2-3% activity of intact calpain 1 Ca\(^{2+}\)-dependent activity upon the binding of two Ca\(^{2+}\) ions. The Ca\(^{2+}\)-dependent activity of the mini calpain 2 (domain I-II) is even weaker (0.001% of intact calpain 2) compared with the mini calpain1 (domain I-II) (Moldoveanu et al.,
Neither the mini calpain 1 nor the mini calpain 2 can be inhibited by calpastatin (Moldoveanu et al., 2003).

Fig. 2: Ribbon structure of human calpain 2 in the absence of calcium

From the website: http://ag.arizona.edu/calpains/

Picture can also be viewed from protein data bank: http://www.rcsb.org/pdb/cgi/explore.cgi?job=graphics&pdbId=1DKV&page=&pid=6246970523863
Calpains in Nervous System Disease

The necrotic death induced by intraneuronal influx of calcium and consequent activation of calpains can be protected by calpain inhibitors (Wang, 2000). It also was reported that calpain mediated necrotic death resulted in the loss of Cornu Ammonis 1 neurons after ischemia (Yamashima, 2000). Activation of proteolytic systems, including calpains, in early stages promotes beta-amyloidogenesis, neurofibrillary pathology, and neurodegeneration in Alzheimer disease (Nixon, 2000). That the increased expression of calpain in activated glial/inflammatory cells participating in myelinolysis and loss of calpain substrates in axon and myelin (Banik and Shields, 1998; Shields and Banik, 1999), leads to the suggestion that calpains play an important role in some autoimmune demyelinating diseases (Schaecher et al., 2001).

In addition to the studies of the only native inhibitor calpastatin, a range of peptidomimetic calpain inhibitors and nonpeptide inhibitors to calpain 1 and calpain 2 were discussed (Donkor, 2000) for the potential treatment value.

Calpains and Their In vivo Protein Substrates

Calpain 1 and calpain 2 can act on a wide range of substrates and researchers showed that limited proteolysis may alter the properties of some proteins during in vivo calpain activation (Suzuki and Sorimachi, 1998). There are several experiments showing that some proteins, including á-fodrin and nebulin (Siman et al., 1984), can be degraded by calpains in live animals, and the degradation of á-fodrin and nebulin could be stabilized by inhibition of calpain 2 in intact skeletal muscle (Huang and Forsberg, 1998). Ever since the early study of calpains, it has been known that classical calpains prefer
proteins as substrates, and have poor activity on peptide substrates (Murachi, 1983). It was suggested that calpain 1 recognized certain secondary structures adjacent to cleavage sites (Wang et al., 1989). It is likely that calpain 3 acts on different physiological substrates, hence there is a different role of calpain 3 compared with the classical calpain 1 and calpain 2 (Sasaki et al., 1984).

It seems that limited cleavage by calpains can affect apoptosis pathways in different stages, in some cases independent of the caspase pathways. Calpains appear to cleave many apoptotic factors, including caspases and Bid in human melanoma cells (Mandic et al., 2002). Calpains, not caspases, produce MPP(+) -induced Bax cleavage in the neuronal cell line MN9D (Choi and Lee, 2001), and proapoptotic Bax N-terminal cleavage into apoptotic p18 in Jurkat T cells (Gao et al., 2000), Bcl, actin proteolysis and DNA fragmentation in chicken ciliary ganglia neurons (Villa et al., 1998) and the upstream of caspases in the radiation-induced apoptosis case Burkitts' Lymphoma cell line BL30A (Waterhouse et al., 1998). In some cases, both caspase and calpain were involved in ionomycin-triggered apoptosis though Bcl-2 family members, as preincubation with the calpain inhibitor acetyl-calpastatin 27-peptide and the caspase inhibitor Z-DEVD-fmk could prevent the apoptosis (Gil-Parrado et al., 2002).

**Calpains-Mediated Cellular Processes by Limited Cleavage of the Substrates**

Calpain 1 and calpain 2 are intracellular proteases acting on a wide range of protein substrates by with limited cleavage (Wang and Yuen, 1999). This limited cleavage may be a regulatory aspect of signal transduction pathways (Glading et al., 2002). Even some early studies showed that protein kinase C could be activated by
calpains through limited proteolysis, enhanced by phospholipids and diacetyl glycerol in the KM3 cell line (Ase, 1989). Persistent activation of G-protein signal pathways via calpain-mediated limited cleavage of Gs alpha subunit was observed in rat pituitary cells (Sato-Kusubata et al., 2000). Calpains can also act indirectly on Gq signaling in SW480 and HEK293 cell lines by degradation of beta-catenin and disruption of Wnt/beta-catenin pathway (Li and Iyengar, 2002). Calpain can trim signal transducer and activator STAT 3 and STAT 5 at the carboxyl-terminal domain, in vivo or in vitro. This results in the generation of dominant-negative forms of STAT (Oda et al., 2002). Nuclear cyclin-dependent kinase inhibitor p27 can also be degraded by calpain through a MAP kinase-dependent process (Delmas et al., 2003).

Migration and proliferation of fibroblasts in the healing process is promoted by Epidermal Growth Factor receptor (EGFR) which can trigger pathways activated through calpain triggered proteolysis (Shiraha et al., 1999). In CD4(+) thymocytes, the common cytokine receptor gamma chain can be cleaved at a specific site by calpain 1 and its direct interaction with calpain small subunit was observed (Noguchi et al., 1996). The calpain 1 cleavage of phosphotyrosine phosphatase 1B, and consequently its subcellular relocation from membrane to cytosol, was correlated with the platelet transition from reversible aggregation to irreversible aggregation in platelet-rich plasma (Frangioni et al., 1993). Also in platelets, a C-terminal 200-kDa fragment was generated from talin by calpain-mediated limited cleavage during the integrin induced signal cascade (Inomata et al., 1996). Further studies of calpain 1 gene knockout in platelet suggested that the ablation of calpain 1 caused significant reduction in platelet aggregation and clot retraction by
impaired tyrosine phosphorylation on several proteins including the beta3 subunit of \( \alpha \) IIb \( \alpha 3 \) integrin (Azam et al., 2001).

Calpain 1 and calpain 2 have been implicated in cell spreading by modification of cell-substratum adhesion sites (Glading et al., 2002). Promotion of adherent cell locomotion \textit{via} cell rear-end detachment involves many adhesive regulators including myosin-mediated cell contractility, tyrosine phosphorylation, rho, calcium fluxes and calpain (Cox and Huttenlocher, 1998). Cleavage of tyrosine phosphatase 1B by calpain resulted in its relocation to the sites of focal contact previously inaccessible to the endoplasmic reticulum-associated native phosphatase (Rock et al., 1997). Clustering of leukocyte integrins was regulated by calpain mediated events through intracellular Ca\(^{2+}\) flux activation (Leitinger et al., 2000). The calpains were activated not only by calcium fluxes, but also by signaling cascades including Protein Kinase C (PKC), tyrosine kinase-, and adhesion molecule-signaling events (Sato and Kawashima, 2001). Some platelet studies showed that calpain-induced cleavage of proteins was required for integrin-induced formation of focal adhesions and actin filament reorganizations with the activation of both Rac1 and RhoA (Fox, 1999). The adhesive and signaling functions of integrin alpha IIb beta in platelets were activated by calpain-mediated cytoskeletal changes (Schoenwaelder et al., 2000). Further investigations demonstrated that calpains cleaved RhoA to generate a dominant-negative form that inhibited integrin-induced actin filament assembly and cell spreading (Kulkarni et al., 2002). Other studies proposed that calpain 1 was activated by beta1 and beta3 integrin-induced signaling (Reddy et al., 2001). This contrasts with the previous suggestion that calpain-mediated cleavage occurs before integrin-induced signaling (Schoenwaelder et al., 2000), and that calpain inhibitors
were able to prevent the cleavage of the α3 integrin cytoplasmic domain, indicating that calpain is required during apoptosis in human umbilical vein endothelial cells (Meredith et al., 1998).

Recently there was a report that local calcium transient can slow axon outgrowth through calpain-dependent manner and again related to integrin-mediated events (Robles et al., 2003).

**Summary of calpain 1 and calpain 2:**

Although the *in vivo* activation mechanism of calpains 1 and 2 remains unclear, the limited cleavage of protein substrates by classical calpains mediate down-regulation or up-regulation of many cellular process, including spreading, integrin-induced cell adhesion, migration, activation/inactivation of nuclear transcriptional factors and cytoplasmic signaling molecules. These molecules and cellular process may offer better understanding in the areas including inflammation, wound repair, tumor progression and some neural diseases.

**Calpain Knockout**

The knockout of both the murine calpain 4 gene copies, encoding the small subunit common to calpains 1 and 2 proteins, leads to embryonic death at day 9 (Zimmerman et al., 2000), but did not affect survival and proliferation of cultured embryonic stem cells or embryonic fibroblasts (Arthur et al., 2000).
Calpain 3: Cause of LGMD2A, and Tentative Mechanism

Among the other calpain members, calpain 3 and calpain-10 have attracted many laboratories due to their involvement in human diseases.

Mutations in the Capn3 gene, encoding calpain 3, cause the human disease limb girdle muscular dystrophy type 2A (LGMD2A) (Richard et al., 1995; Fougerousse et al., 1998). And in some populations most of LGMD2 disease results from calpain 3 gene mutations (Pogoda et al., 2000). Capn3 is located in 15q of human chromosome 15 (Minami and Nishino, 1999). The natural mutations resulting in the loss of calpain 3 proteolytic activity causes LGMD2A (Richard et al., 2000).

Calpain 3, also named p94, was purified and its autoproteolysis characterized (Kinbara et al., 1998). Calpain 3 has a calmodulin-like Ca$^{2+}$-binding domain similar to domain IV of calpains 1 and 2, although the autolysis did not depend on the presence of Ca$^{2+}$ (Kinbara et al., 1998). Calpain3 can undergo rapid autoproteolysis (Kinbara et al., 1998; Herasse et al., 1999) when expressed in muscle tissue. Two suggested autolytic sites (Federici et al., 1999) have been investigated.

Alpha-fodrin is believed to be one of the calpain 3 substrates (Ono et al., 1998; 1999). Calpain 3 can bind to connectin/titin (Ono et al., 1998; Richard et al., 1999) and co-purified with titin (Sorimachi et al., 2000). The binding to titin probably suppressed calpain 3 activity (Sorimachi et al., 2000).

In one case, 97 distinct pathogenic calpain 3 mutations have been identified (4 nonsense mutations, 32 deletions/insertions, 8 splice-site mutations, and 53 missense mutations) in LGMD2A patients (Richard et al., 1995, 1999; Ono et al., 1998).
In the other types of muscular dystrophy, secondary loss of calpain 3 activity could be a downstream effect. Tibial muscular dystrophy (TMD) results from the mutations of the titin protein adjacent to the calpain 3 binding site (Haravuori et al., 2001). A mouse model was identified with mutations in titin resulting in loss of interaction with calpain 3. These mice develop severe and progressive muscular degeneration (Garvey et al., 2002). Transgenic mice expressing an catalytic mutant of calpain 3 (active site Cys to Ser, C129S) had significantly decreased grip strength (Tagawa et al., 2000).

A muscle-specific inducible system expressing active calpain 3 at different levels indicated that decreased calpain 3 activity significantly increased myogenin, which was a highly specific transcription factor involved in myoblast fusion (Dargelos et al., 2002). One yeast two hybrid screen demonstrated that calpain 3 interacted with titin through an IS2 region only in calpain 3 of calpain family members (Sorimachi et al., 1995; Jia et al., 2001). Further studies showed that titin can bind to calpain 3 through two sites encoded by titin M-is7 exon, with the one at the N-terminal sometimes missed due to alternatively splicing in different muscle tissues (Kinbara et al., 1997).

Considering its pathogenic significance, little is known about the function of calpain 3. Several mechanism have been proposed for LGMD2A, including loss of protein degradation in LGMD2A patients (Richard et al., 1995), toxicity related to accumulation of mutant calpain 3 (Tagawa et al., 2000), or destabilization of cytoskeletal components (Balcerzak et al., 1995). Others reported that there was no relationship between calpain 3 inactivation and the degradation of cytoskeletal components (Wang and Yuen, 1999). Other pathogenic factors may include loss of proteolytic activity of
calpain 3 mutants (Ono et al., 1998, 1999; Richard et al., 2000), rapid autolysis and degradation of mutant calpain 3 (Spencer et al., 1997), IêBá accumulation due to calpain 3 deficiency, resulting in apoptosis (Baghdiguian et al., 2001), or increase of myogenin after loss of calpain 3 proteolytic activity (Dargelos et al., 2002). There was some contradiction among these proposals.

Summary of Calpain 3

Calpain 3 binds to titin and its proteolytic activity is important to its physiological function. Several downstream events affected by calpain 3 proteolytic activity have been proposed. The physiological role of calpain 3 can likely be explained in the context of the interaction with titin as discovered by a yeast two hybrid screen (Kinbara et al., 1997; Sorimachi et al., 2000). Because LGMD2A patients only present the symptoms starting from the 2nd decade of life (Wang and Yuen, 1999), the developmental factors affected by active calpain 3 cannot be factors essential to the survival of myo lineage cells (Tagawa et al., 2000; Spencer and Guyon, 2002). We believe that the early stage of muscular development does not require active calpain 3 or active calpain 3 plays a minor role; however, the later stage of muscular growth and development need some factors to be cleaved in muscle tissue by the proteolytic capable calpain 3. A differential screen may help find the crucial factors expressed around puberty and regulated by calpain 3 during later muscular development stages.
Other Conventional Calpains

Calpain 9, also named nCL-4, is a digestive tract-specific calpain (Lee et al., 1999). It was first cloned in 1998, and found to be 50-55% homologous to other mammalian calpain large subunits (Lee et al., 1998). Later, calpain 9 was found markedly induced in the pituitary after estrogen treatment (Duan et al., 2002). Calpain 9 appears to be a gastric cancer suppressor (Huang and Wang, 2001). It is suppressed or depleted in many gastric cancer cell lines (Yoshikawa et al., 2000), and expression of its antisense RNA induced anchorage-independent growth and tumorigenesis in mice (Liu et al., 2000).

Capn11 mRNA is exclusively expressed in spermatocytes and only during the later stages of meiosis (Dear and Boehm, 1999).

Calpain 12, located on mouse chromosome 7, is mainly expressed in the cortex of the hair follicle with 3 different splicing variants (Dear et al., 2000).

The Unconventional Calpains

The preceding sections dealt largely with calpain family members containing the calcium binding domains represented by domains DIV and DVI in calpain 1 and calpain 2. Other calpains containing no calcium binding domains with EF-hand motif are called unconventional calpains (Huang and Wang, 2001). Calpain 5 and 6 were discovered to be similar members of the calpain gene family (Matena et al., 1998). The C-terminal region of calpain 5 and calpain 6 lack homology to the calmodulin-like domain in the other mammalian calpains (Dear et al., 1997). By definition, they are both unconventional calpains. Both calpain 5 and calpain 6 exhibit significant homology to the C. elegans
protein encoded by the tra-3 gene, which is involved in nematode sex determination (Hodgkin, 1986) and regarded to be the homolog to calpain 5 in higher organisms (Dear et al., 1997). Calpain 5, closely linked to the calpain 1 gene, is localized on human chromosome 11 or mouse chromosome 7, but calpain 6 is localized on the human X chromosome (Matena et al. 1998). Calpains 5 and 6 have different mRNA expression sites during mouse embryogenesis, with capn5 expressed in developing thymus, sympathetic and dorsal root ganglia, Capn6 in skeletal and heart muscle (Dear and Boehm, 1999). Calpain 6 lacks critical active site residue and may not be proteolytic active (Dear et al., 1997). It is a tempting idea that calpain 6 may be a dominant negative mutant regulating calpain 5 function by competing for binding to calpain 5 substrates.

Calpain 10: Its Association with Type 2 Diabetes, Its SNPs

**Type 2 Diabetes and Insulin Resistance**

Insulin resistance is a key factor in the pathogenesis of type 2 diabetes mellitus, affecting at least 5% of the human population (Hager et al., 1995; Haffner and Miettinen, 1997; Ovalle and Azziz., 2002), and a co-factor in postchallenge hyperglycemia in older population (Chang and Halter, 2003). It seems that various gene mutations are known to play a major role in impaired insulin sensitivity (Hager et al., 1995; Bruning et al., 1997; Pedersen, 1999, Ueda et al., 2000). Type 2 diabetes seems overall to be a polygenic disease (Galli et al., 1996; Accili et al., 2001; Froguel and Velko, 2001). By fine-mapping and positional cloning, a polymorphism UCSNP-43G/A of calpain-10 was identified association with insulin resistance of glucose disposal (Horikawa et al., 2000; Stumvoll et al., 2001). Interestingly, insulin resistance of a common polymorphism (Gly972Arg) in
the insulin receptor substrate 1 (IRS-1), long believed to be a plausible candidate gene, is a weak factor related to type 2 diabetes (Stumvoll et al., 2001).

**Positional Cloning of Calpain 10 and Its Preliminary Studies**

Calpain 10 was initially discovered to be associated with type 2 diabetes by positional cloning of a gene located in the NIDDM1 region in chromosome 2q in several populations (Horikawa et al., 2000; Cox, 2002). This positional cloning was hailed as a landmark study, due to the overall difficulties in investigating polygenic diseases (Patterson, 2000; Menzel, 2002). SNP-43(G/G) in the calpain-10 gene is thought to be associated with insulin resistance and reduced skeletal muscle calpain-10 transcripts in Pima Indians (Baier and Permana, 2000; Yang et al., 2001).

Calpain 10 is an unconventional calpain present in many tissues including lens, retina, heart and skeletal muscles, as determined by western blot analysis (Ma et al., 2001; Reed et al., 2003) and by northern blotting (Horikawa et al., 2000). Although some calpain-10 was detectable in the water-soluble protein fraction of these tissues, it was preferentially found in the water-insoluble fraction (Ma et al., 2001).

**Single Nucleotide Polymorphism in Calpain 10 Gene**

The calpain-10 association with type 2 diabetes depends on the population investigated (Elbein, 2002) and there are differences in the distribution of CAPN10 susceptibility variants between African and non-African populations (Fullerton et al., 2002).
Variation in the calpain-10 gene (CAPN10) has been shown to be associated with type 2 diabetes in Mexican Americans and in at least three northern European populations (Horikawa et al., 2000). Studies in nondiabetic Pima Indians showed that SNP-43 in CAPN10 was associated with insulin resistance, and individuals with the G/G-genotype had significantly higher fasting plasma glucose and 2-h insulin concentrations after a 75-g oral glucose tolerance test (Lynn et al., 2002), but not in German population (Stumvoll and Haring, 2001).

The SNPs genetic variation in the CAPN10 gene influenced blood glucose levels in nondiabetic British population (Lynn et al., 2002). Individuals bearing SNP-43 with the G/G genotype were more likely to have diabetes than those with the A/G or A/A genotype in African American population (Garant et al., 2001).

The 112/121 haplotype combination defined by the SNP-43, -19, and -63 alleles in the calpain-10 gene is associated with type 2 diabetes in Mexican Americans (Horikawa et al., 2000), but not in the Samoans of Polynesia (Tsai et al., 2001) and not in Japanese (Horikawa et al., 2003). SNP-43 and SNP-44 affects not type 2 diabetes but significantly increased serum cholesterol levels in Japanese (Daimon et al., 2002).

There are significant frequency differences of SNP-41,44,19,63 alleles in Chinese in comparison with other ethnic groups (Sun et al., 2002). Variations of calpain-10 gene sequence impacts the metabolic parameter levels related to type 2 diabetes mellitus in the Chinese population, depending on the haplotypes as well as the haplotype combinations (Xiang et al., 2001). However, there is no association with some groups in northern China (Sun et al., 2002). SNP-43 in the CAPN 10 gene might be one of the genetic factors
contributing to hypertension and diabetes mellitus (Li and Iyengar, 2002), and SNP-44 in CAPN 10 has an impact on fasting plasma glucose levels, and after glucose challenge (Wang et al., 2002).

There was a positive association of the calpain-10 121/121 haplotype combination of SNPs 43,19 and 63 with type 2 diabetes in a Polish population (Malecki et al., 2002). The SNP-43,56,63 Variation in calpain-10 gene was not associated with type 2 diabetes in the Finnish population (Fingerlin et al., 2002) or German populations (Hinney et al., 2002). However, there was also a conflicting report that SNP-43, SNP-63, and the haplotype combination SNP-44/43/19/63 1121/1121 were associated with type 2 diabetes in the Finnish population (Orho-Melander et al., 2002). Haplotype combination 1112/1121 (UCSNP44, -43, -19, and -63) appeared to increase the risk of type 2 diabetes in a south Indian population, despite its low frequency (Cassell et al., 2002). The frequency of the 112/121 at-risk haplotype of CAPN10 is too low among Scandinavians to make a conclusion (Rasmussen et al., 2002).

Each variant separately and as haplotype combinations of SNP-43, SNP-19, and SNP-63 were not associated with type 2 diabetes in some Caucasian populations, although both SNP19 and SNP63 increased fasting and/or post-glucose challenge insulin levels (Elbein et al., 2002).

There are reports that SNPs in CAPN10 may be associated with other diseases, including insulin-glucose related metabolic abnormalities other than type 2 diabetes. Deletion/insertion of SNP 19 in the calpain-10 gene is associated with reduced â3-adrenoceptor function in obesity (Hoffstedt et al., 2002a). The SNP-44 allele is associated with polycystic ovary syndrome (PCOS) in the Spanish population (Gonzalez et al.,
2002), but not PCOS in the UK (Haddad and Evans, 2002). The 112/121-haplotype combination was associated with an approximate 2-fold increase in risk of PCOS in both African Americans and whites in the USA (Ehrmann et al., 2002).

The C allele of SNP45 is associated with idiopathic hirsutism, and SNP43 influences the hirsutism score (Escobar-Morreale et al., 2002).

Maximum microvascular hyperaemia was increased with G/G genotypes at SNP-43 compared to the combined group of subjects G/A and A/A genotype at SNP-43, and the minimum microvascular resistance was reduced. However, the two groups did not differ in terms of anthropometric measures, blood pressure, insulin resistance or glucose (Shore et al., 2002).

Regarding SNP-43, the G/G genotype had two-fold higher basal and insulin-stimulated glucose oxidation rates as compared with AA/AG genotypes in Sweden (Hoffstedt et al., 2002b).

Summary on Calpain-10

In general, the physiological role of calpain-10 associated with type 2 diabetes has been much less studied than of calpain 3 to LGMD2A. Many of the human genetic analyses on the association between type 2 diabetes and single nucleotides polymorphism (SNP) in capn10 depend on different ethnic groups and geographies when investigated.

Current studies on the SNP variations of calpain-10 do not support it to be the major cause of type 2 diabetes according to the various reports, but it is involved in glucose metabolism and insulin resistance, at least in certain SNP genotypes in some human populations (Baier and Permana, 2000). Thus, one review proposed that the
cumulative effects of a combination of variants, rather than variation at a single site inside the intron, increases the susceptibility to type 2 diabetes (Cox, 2002).

Because all of the current human genetic linkage assays focused on the SNPs in the introns of calpain10 (Cox, 2002) except in one case (Horikawa et al., 2003), whether genetic variations in the coding region of calpain-10 affects the susceptibility to type 2 diabetes still remains an open question.

One unique property of calpain-10 is that it is the only mammalian calpain with tandem DIII domains (Sorimachi and Suzuki, 2001). A hypothesis underlying the current studies in our lab is that these tandem DIII domains hold the key to function of calpain-10. It was known that calpain 1 and calpain 2 share a wide range of common substrates (Wang and Yuen, 1999), the potential functional redundancy of the calpain genes makes it difficult to study the physiological role of individual members when they are expressed ubiquitously or exist in the same cells. There is the other disadvantage that higher organisms usually possess diploid genomes. Experimental animals with double recessive alleles can be obtained by knockout and subsequent crossbreeding, but it is very time consuming.

With the considerations discussed above, it appears that the eukaryotic slime mold Dictyostelium discoideum may provide a useful model organism for calpain research, due to its fast doubling time and relative ease of genetic manipulation. The proposal is that certain function(s) of calpains may be elucidated in this lower organism, it may provide useful clues about calpain-10 function before research is carried out in mammalian organisms. Based on Blast searches through the Dictyostelium databases, EST clones with tandem domains homologous to domain III in mammals were the only candidate
genes found. The *cplA* gene was found to encode Cpl, a calpain-like protein with tandem DIII domains (Huang et al., 2003). Thus, it appears more similar to calpain-10 than to the other mammalian calpains.

**Yeast Two Hybrid Screen in Calpain Research**

The yeast two hybrid system can be used in two aspects of calpain research. One is looking for calpain substrates. The other is looking for calpain protein-protein interaction partners.

Since its invention (Fields and Song, 1989), the yeast two hybrid (Y2H) screen has been applied to many proteins to find their interaction partners despite some limitations of the technique (Toby et al., 2001). Two extreme cases may be cited. The expression of full length prey cDNA can be toxic to yeast, hence leading to the loss of potential clones (D'Orazi et al., 2001). Although it has been proposed (Richard et al., 1995) that the Y2H system may help to identify calpain substrates by using a catalytically inactive mutant as the bait, in theory and in practice this approach does not appear to be feasible. First, action of the Y2H system is based on the association of bait and prey proteins. Any Ka for protein-protein interaction lower than $10^6$ will be considered as a non-specific, weak interaction. Weak interactions will result in either no signal when selected in high stringency conditions or too many false positives when selected in low stringency conditions. Therefore, a high Ka for protein-protein interactions is the key to identifying positive clones in Y2H screens. Unfortunately, it is unlikely that for most enzyme-substrate interactions the apparent association constant would be greater than $10^6$. In practice, one study designed to identify substrates for caspase3 resulted in only
gelsolin (Kamada et al., 1998), although many more proteins were already known to be caspase 3 substrates. A trial to screen the substrates of calpain 2 by Y2H screen resulted in only 29 positive clones interacting with DII catalytic domain, before further studies (Jiang et al., 2002) identified approximately 100 proteins believed to be cleaved by calpain 1 and 2 (Wang and Yuen, 1999). The Y2H screen for calpain interaction partners might give too many "positive" clones, if low stringent selection is used (Jiang et al., 2002).

Our hypothesis is that the tandem DIII domains in Cpl or calpain-10 may be crucial to their function. Moreover, these two proteins are composed of putative catalytic domains plus the tandem DIII domains. Therefore, it seems reasonable that the latter would constitute a "targeting" domain, guiding the localization of the catalytic domain to its physiologic substrate(s).

**Dictyostelium Discoideum as A Model System for Studying Calpain 10 Function**

*Dictyostelium discoideum* grows in unicellular form under optimal growth conditions. The individual cells of slime molds release extracellular messenger cAMP when nutrient depletion occurs, and start the multicellular developmental procedure through cell surface cAMP receptor-mediated signaling pathways (Parent and Devreotes, 1996). Moving along by extracellular cAMP gradient signals, the whole unicellular population migrates to a central site to aggregate, develop and form a multicellular fruiting body in the final aggregation stage (Kessin, 2001). The slime molds can be helpful to understand some physiological processes in higher organisms. Especially
informative are studies of those proteins in the slime molds that are homologous to their counterparts in higher organisms.

*Dictyostelium* amoebas, like mammalian macrophages, takes up fluid by macropinocytosis by specialized endosomes associated with actin filaments and microtubules (Clarke et al., 2002). N-ethylmaleimide (NEM)-sensitive factor (NSF) is an essential protein required during bulk fluid transport processes such as macropinocytosis (Thompson and Bretscher, 2002). The endocytosis vesicles bind specifically to F-actin filaments (Lee and Knecht, 2002) through Scar-mediated polymerization of the actin (Seastone et al., 2001) and coronin equally (Hacker et al., 1997). When amoebas are cultured under vegetative conditions, macropinocytosis is induced to meet nutritional requirements depending on PI3-kinase signaling to the actin cytoskeleton. A dominant-negative PI3-kinase in transformed fibroblasts abolishes macropinocytosis, and dominant-positive P13K in non-transformed fibroblasts is sufficient to induce macropinocytosis (Amyere et al., 2002) through the PI3 kinase downstream effector, protein kinase B (PKB/Akt) (Rupper et al., 2001). Disruption of RasGEFb causes *Dictyostelium* to move unusually rapidly, but the amoebae lose the ability to macropinocytose, and therefore are unable to grow in vegetative medium (Wilkins et al., 2000). In addition to the major fluid phase macropinocytosis uptake pathway, there is a micropinocytic pathway in *Dictyostelium* (Neuhaus et al., 2002).

**Flotillin in The Insulin-Induced Glucose Transportation**

Flotillin was first cloned as a protein homologous to epidermal surface antigen (ESA) and caveolae-associated integral membrane proteins (Bickel et al., 1997). It has
been reported that flotillin 1 and 2 bind to Caveolins 1 and 2 to form a stable hetero-
oligomeric complex subject to co-immunoprecipitation (Volonte et al., 1999), but the
association with caveolae and co-IP with caveolins was not confirmed in another
publication (Salzer and Prohaska, 2001). The N terminal PHB domain of flotillin-1 is
sufficient to target a heterologous protein to the plasma membrane and the highly
conserved Cys34 residue of PHB domain is required for membrane targeting (Morrow et
al., 2002). Flotillin can form a ternary complex with the insulin receptor substrates cbl
and CAP and the overexpression of CAP without SH3 domain can attenuate insulin
induced glucose metabolic effects (Baumann et al., 2000; Czech, 2000).
MATERIALS

*Dictyostelium discoideum* AX-3 strain, 3T3-L1 pre-adipocyte, and human β-actin cDNA were obtained from ATCC (ATCC#: 28368).

Mouse anti-Myc antibody were from Invitrogen.

Anti-mouse 2nd antibody was purchased from Sigma.

A rabbit anti-Cpl antibody against the peptide antigen (DFQNEMVFTKTSNWEKRND) was custom prepared by Bethyl Laboratories.

Cpl EST clones derived from slug-stage libraries were kindly supplied by the *Dictyostelium* cDNA Project, Tsukuba, Japan. The clones were: SLC473 (GenBank accession number AU060977) and SSC516 (GenBank accession number AU071783).

Match maker 3 yeast two hybrid screen kit, human fetal liver library for yeast two hybrid screen, yeast minimal SD base, yeast SD dropout SD-Leu-Trp, and SD-Leu-Trp-Ade-His were purchased from Clontech.

*Pfu* PCR polymerase was from Stratagene.

T4 DNA ligase was obtained from Invitrogen.

CEQ DCTS sequence kit for CEQ 2000 automaton was from Beckman Coulter.

SuperSignal West Pico Chemiluminescent substrate was purchased from Pierce.

PGEX-4T1 vector for GST fusion expression was obtained from Amersham-Pharmacia.

The DNA random primer labeling kit was from Roche.

Leupeptin and Pepstatin were supplied by Chemicon.
Bacto yeast extract, Bacto Tryptone, Bacto Peptone and granulate agar were obtained from Difco.

Electrophoresis grade agarose was from Invitrogen.

SeaPrep agarose was from BioWhittaker Molecular Applications.

Low melting agarose was purchased from Gibco BRL.

Bacteriological peptone was obtained from Oxoid.

Dithiothreitol thrombin, DEAE-sepharose CL-6B, and Phenyl-sepharose CL-4B were obtained from Sigma.

BioGel A1.5m was from Bio-Rad.

All the restriction enzymes used in the studies were purchased from Invitrogen or Promega.

Integrated DNA Technologies provided DNA primers used in the studies.

EcoRI/NotI adapters were from Invitrogen or Stratagene.

Pre-stained protein markers were obtained from Bio-Rad.

Gene Pulser Cuvettes were supplied by Bio-Rad.

DH5alpha subcloning efficiency chemical competent E. coli., and DH10B electroporation competent E. coli. were from Invitrogen.

The Micro polyA purification kit was obtained from Ambion.

Centricon tube protein concentrators were purchased from Amicon.

Special Acknowledgments:

SLC473 and SSC516 Cpl EST clones were provided by The Dictyostelium cDNA project in Japan, supported by the Japan Society for the Promotion of Science, and the
Ministry of Education, Science, Sports and Culture of Japan. Where indicated, contig sequence data were produced by the *Dictyostelium discoideum* or *Entamoeba histolytica* Sequencing Groups at the Sanger Institute and can be obtained from http://www.sanger.ac.uk/Projects/. Mouse GST-fotillin plasmid for pulldown assays was a gift from Dr. Alan R. Saltiel, the University of Michigan Medical Center. pBM272 yeast expression vector induced under Gal was a gift from Dr. Robert Trumbly of the Medical College of Ohio.
METHODS

1. **Dictyostelium culture.** Stock cultures of *Dictyostelium discoideum* strain Ax-3 amoebae were maintained at room temperature (23°C to 25°C) in HL5 media, with a shaking speed of 120 rpm.

2. **Purification of Cpl.**

   **Culturing AX-3.** Ax-3 *Dictyostelium discoideum* were cultured at ambient temperature in SM medium containing autoclaved *Escherichia coli*, to a density of 5 to 6 X 10^6 amoebae/ml.

   **Harvesting and lysis of Dictyostelium.** All purification steps were carried out at cold room temperature (5°C to 8°C). Cpl containing fraction were identified by immunoblot analysis. The standard purification buffer was buffer A: 50 mM imidazole-HCl, 200 μM Na_3VO_4, 6 mM EGTA, 50 μM Leupeptin, 15 μM Pepstatin A, 500 μM PMSF, and 2 mM dithiothreitol, pH 7.4 measured at room temperature. Ten liters of culture were centrifuged at 1200 X g for 15 min. The pellet (50 to 60 ml of packed amoebae) was washed twice with 60 ml of development buffer: 5 mM Na_2HPO_4, 5 mM KH_2PO_4, 1 mM CaCl_2, and 2 mM MgCl_2, pH 6.5. The sedimented cells were lysed in 120 ml of lysis buffer (buffer A containing 150 mM NaCl and 1% NP-40 detergent) and centrifuged at 12,000 X g for 30 min.

   **The 1st DEAE chromatography.** The 12,000 X g supernatant was diluted to an ionic strength equal to 50 mM imidazole + 50 mM NaCl by addition of two volumes of buffer A, and mixed with 30 ml of packed, equilibrated DEAE-Sepharose. After 3 h of
equilibration with occasional stirring, the slurry was applied to a large Buchner funnel, and washed sequentially with 200 ml each of buffer A, and buffer A containing 100 mM NaCl. The gel slurry was packed into a chromatography column, and eluted with buffer A containing 320 mM NaCl, collecting 0.5 ml each fraction.

**Bio-Gel gel filtration:** The Cpl-containing fractions detected by immunoblot analysis (8-16 ml) were pooled and transferred to a dialysis tube. The sample was concentrated to less than 3 ml by dehydrating against dry polyethylene glycol (cut off MW = 3,000). The concentrated sample was then applied to an 80 ml Bio-Gel A-1.5m gel filtration column pre-equilibrated with buffer A containing 100 mM NaCl. The flow rate was 4 to 6 ml per h, and 1.6 ml fractions were collected.

**Gradient DEAE chromatography.** An 8 ml DEAE column was packed and equilibrated with buffer A (50 mM Imidazole 50 mM NaCl pH 7.4). Pooled Cpl fractions from the Bio-Gel column (10 ml) were diluted with an equal volume of buffer A and loaded onto the DEAE column. Cpl was eluted with a 40 ml 0.2 to 0.5 M NaCl gradient in buffer A.

**Phenyl-sepharose chromatography.** Cpl fractions from the second DEAE column were adjusted to 0.65 M NaCl, and applied to a 3 ml Phenyl-Sepharose column equilibrated in the same buffer. The flow through sample was re-loaded onto the column three times. This step removed contaminating proteins that bound to the Phenyl-Sepharose column below 0.65 M NaCl. Cpl did not bind. The 0.6 M NaCl pre-adsorbed elution was then adjusted to 2 M NaCl, and passed through a 0.5 ml column of Phenyl-Sepharose equilibrated in buffer A plus 2 M NaCl. The gel was washed with 5 ml of equilibration buffer, and the Cpl was eluted with a descending NaCl gradient in Buffer A:
2 M to 0 M NaCl, in a total volume of 20 ml. Following the gradient elution, tightly absorbed proteins were eluted with buffer A containing 1% NP-40. Fractions of 0.5 ml were collected. Aliquots of fractions containing Cpl were concentrated 40 to 60-fold in a Centricon YM-30 centrifugal concentration chamber and stored at 4°C. The overall yield was 90 μg of purified Cpl protein from 5 X 10^10 amoebae (Table I).

3. Hydrodynamic properties of Cpl. *Dictyostelium* were lysed by homogenizing in two volumes of lysis buffer (Buffer A containing 150 mM NaCl, 1.5% NP-40). After incubation on ice for 60 min, the lysis mixture was passed through a 25 gauge needle, and centrifuged at 12,000 X g for 15 min. The supernatant was applied to sucrose gradient ultracentrifugation, or gel filtration on a calibrated Bio-Gel column. Cpl 77 KDa immunoreactivity in fractions was determined by immunoblotting. Relative S_{20,w}, Stokes radius, and molecular mass were determined. Sedimentation coefficients and Stokes radii of protein standards were: bovine liver catalase, 11.3 S, 5.1 nm; bovine serum albumin, 4.6 S, 3.6 nm; whale myoglobin, 2.0 S, 1.87 nm. The partial specific volume of Cpl was estimated from its amino acid content.
Table I. Purification Summary of Cpl.

The starting material was 10 liters of culture containing $5 \times 10^{10}$ *Dictyostelium* amoebae. Specific activity was determined by caseinolytic activity assay. A unit of protease activity generates 1 ng of TCA-soluble casein fragments per min under the standard assay conditions at room temperature.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Protein (ml)</th>
<th>Volume (ml)</th>
<th>Total Protein (mg)</th>
<th>Units/ml</th>
<th>Total Units</th>
<th>Units/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEAE-Sepharose</td>
<td>4.50</td>
<td>8.00</td>
<td>36.00</td>
<td>180</td>
<td>1440</td>
<td>40</td>
</tr>
<tr>
<td>Bio-Gel A1.5m</td>
<td>0.95</td>
<td>10.00</td>
<td>9.50</td>
<td>130</td>
<td>1300</td>
<td>137</td>
</tr>
<tr>
<td>Phenyl-Sepharose, conc. Fractions</td>
<td>0.35</td>
<td>0.25</td>
<td>0.09</td>
<td>633</td>
<td>158</td>
<td>1810</td>
</tr>
</tbody>
</table>
Sucrose gradient ultracentrifugation. Sucrose gradients were prepared by adding and freezing layers of sucrose concentrations ranging from 25\% to 5\%. All subsequent steps were carried out at 4\(^\circ\)-8\(^\circ\)C. AX-3 lysate centrifuged at 10,000 x g for 10 min was loaded onto the top layer of the thawed sucrose gradient tube. After centrifugation for 15 h at 39,000 rpm in an SW41 rotor, the 4 ml sucrose gradient volume was aliquoted into 19 fractions. Molecular weight was calculated relative to standard proteins by an established method (Siegel and Monty, 1966), decided by the equation:

\[
MW = \frac{[6\delta\zeta N/(1-i\eta)] S_{20,\text{w}} R_s}{S_{20,\text{w}} R_s = 4064 S_{20,\text{w}} R_s}
\]

Protease assay. Prior to assay, chromatography fractions were centrifuged in Centricon YM-30 tubes to remove inhibitors, and resuspended in an equal volume of buffer A without protease inhibitors. In one experiment, Phenyl-Sepharose purified Cpl was concentrated, resuspended in buffer without inhibitors, and then concentrated 20-fold again prior to assay. Calcium-activated caseinolytic activity was determined on chromatography fractions as previously described (Mellgren et al., 1982), using \(^{14}\text{C}\)-methylated casein (1000 cpm/\(\mu\)g) as the substrate. Briefly, fractions were incubated with or without 5 mM calcium acetate at 25\(^\circ\)C with 0.5 mg/ml \(^{14}\text{C}\)-casein. After 1 to 4 h, TCA was added to 2.5\%, and the samples were centrifuged to remove precipitated casein. Radioactivity in the supernatant fraction was counted in a liquid scintillation counter to determine proteolytic release of TCA-soluble peptides from casein. Ca\(^{2+}\)-activated caseinolysis was determined by subtraction of activity measured in the absence of calcium from activity measured in its presence. A unit of protease activity generates 1 ng
of acid-soluble casein fragments per min under the standard assay conditions (TCA, trichloroacetic acid).

**Protein immunoblotting.** Cpl in chromatography fractions was detected by immunoblot analysis, using a rabbit immune serum prepared against a sequence (DFQNMVEFTKTSNWEKRD) predicted to be on a surface loop of the second Cpl D-III domain. Immunoblotting and staining were carried out by established methodology, utilizing alkaline phosphatase conjugated second antibody and indoxyl phosphate/nitroblue tetrazolium visual staining. In some experiments, blots were scanned for the intensity of Cpl staining, using a Bio-Rad scanner and Molecular Analyst software.

**Yeast two hybrid (Y2H) screen.** Yeast bait protein containing calpain-10 tandem DIII domains was cloned into pGBK7 vector and expressed in AH109 yeast for Y2H screening. Five yeast AH109 clones were grown in synthetic media minus Trp (SD-Trp) and analyzed by Western blot, showing a strong immunostaining band utilizing anti myc mouse antibody. Y2H human fetal liver library ready for Y2H screening was purchased from Clontech. Y2H screens were performed according to the Clontech manual.

1. cDNA containing DIIIX2 domains of calpain-10 (the 1096 bp to 2192 bp fragment from human full length calpain-10 cDNA) was subcloned into pGBK7 vector by directional sites *Nco* I and *Pst* I. The plasmid was transformed into the yeast strain AH109. There was no endogenous transcriptional activity by this plasmid alone on SD-Leu-Trp-His yeast selection plates.
2. The AH109 strain containing pGBK7+calpain10 DIIIX2 was grown in SD-Trp media to a density of over 1X10^9 cells for mating. For mating, the bait yeast cells were mixed with 1ml Clontech human liver library in yeast Y187 strains in 50 ml 2xYPDA media. The mixture was grown in 2-liter flask in 30 °C 20-40rpm on the shaker according to the Clontech Y2H library manual.

3. The mating culture was spun down after 20-24 h, then washed in TE buffer and resuspended in 10 ml TE. One ul was plated on SD-Leu plates with dilution factor around 1x10^6 to count the Y187 library cells. Another 1ul was spread to SD-Trp plate with dilution factor around 1x10^9 to count the number of bait yeast. Another 1ul was spread to SD-Leu-Trp plate to count the diploid cell number for the calculation of mating efficiency. Overall, 3x10^6 diploid yeast cells were screened for two-hybrid interaction.

4. The remaining 10 ml was spread to 50 150 mm SD-Leu-Trp-His plates, a low stringent condition, for Y2H screen.

5. Several clones appeared on the selection plate on day 4, but most of the clones appeared after 8 d incubation at 30°C. Numerous tiny clones appeared on the SD-Leu-Trp-His plates beyond 12 d incubation. These were false positive clones as they did not grow when transferred onto SD-Leu-Trp-Ade-His plates.

6. Overall 140 clones appearing on the SD-Leu-Trp-His plates after day 8 growth were picked and streaked onto grid SD-Leu-Trp-Ade-His X-α-Gal plates (i.e., high stringent selection plates). After 3-4 d incubation, several clones stopped growth and many clones continue their growth on high stringent selection plate but with white color. The former was regarded as false positive and the later was regarded as very weak
interaction unable to trigger the expression of LacZ (which is regulated by weaker promoter than reporter genes HIS3 and ADE2). Less than 50% of the clones grew and became blue at different intensity levels on SD-Leu-Trp-His-Ade X-α-Gal plate. Only a few clones turned strong blue.

7. After day 4, those blue clones on the high stringent selection plates were picked up and grown in 2 ml SD-Leu-Trp-His-Ade liquid media and shaken at 30°C until yeast pellets apparently were formed on the bottom of tube. Five μl of liquid culture was spotted onto grid SD-Leu-Trp-His-Ade X-α-Gal plates. Most of clones continued their growth and turned bluish on high stringent selection plates. These bluish clones were picked according to depth of blue color. This step is essentially a replication step to verify the positive clones obtained from last step.

In practice, there was always some difference between the depth of blue color and even strong blue clones cannot maintain the dark blue color after replication. Nevertheless, this LacZ report gene is a useful marker to pick positive clones.

8. Those positive clones, about 40 in the first batch and 30 in the second batch, were grown in SD-Leu-Trp-His-Ade liquid media to harvest enough diploid yeast pellet for plasmid extraction according to the yeast protocol in Current Protocols in Molecular Biology. The extracted aqueous supernatants were subject to PCR reaction.

Seventy PCR products of various sizes were applied to low-melting DNA agarose electrophoresis, and gel slices containing the DNA bands were cut and saved for analysis (see step 9).
Low melting agarose containing the PCR product was melted at 60°C, and 10 µl was mixed with restriction reaction containing *Alu* I enzyme in 20 µl total volume. Digestion was for 4 h at 37°C.

The digests were heated again to 60°C, then loaded onto 2% DNA agarose gels for electrophoresis and photography. The DNA digestion pattern of the 70 clones was compared and sorted. Assuming that identical DNA inserts in the prey library will generate the same *Alu* I cut pattern, there were 15 clones with redundancy and 18 clones that gave unique *Alu* I digestion patterns. The 14 clones with redundancy were selected for DNA sequencing analysis, and later transformed into *E. coli*.

9. Plasmids were prepared from these 14 clones and subjected to sequencing using a Beckman CEQ2000 DNA sequencer. DNA sequences were compared with the GenBank database.

**Yeast protein extraction for co-immunoprecipitation**

Diploid yeast expressing both BD-capn10DIIIX2 and prey protein were cultured in shaking flasks at 30°C to an O.D._550_ of 1.0 before harvesting. For lysis, a yeast pellet:glass beads:lysis buffer ratio of 1:1:2 was used. The mixture was vortexed vigorously 7x1 min in lysis buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 10 mM MgCl₂, 1 mM DTT, 40 µM leupeptin, 20 µM pepstatin, 1 mM PMSF, 10% glycerol, pH 7.5), chilling on ice between intervals. The yeast lysate was centrifuged at 10,000xg for 10 min, and incubated with 9E10 anti-myc antibody which was coupled with protein A agarose beads at 4°C for 120 min. Agarose beads were washed 4x5 min in PBS before boiling in SDS-SPB buffer and further Western blot assay.
DNA gel shift

Oligomer 1: 5’- TT*C T*CT GAG CTA CGG AAG ACT CTC CTC CGC ATC AC -3

Oligomer 2: 5’- GG*C *CTA GTG ATG CGG AGG AGA GTC TTC CGT AGC TC-3

Oligomers were synthesized and annealed in STE buffer (10 mM Tris 50 mM NaCl 1 mM EDTA, pH 7.5) by heating to 94°C 3 min then cooling down slowly to room temperature to form dsDNA oligonucleotides. The oligonucleotides were $^{32}$P labeled by flushing the overhang where the C is marked by an asterisk.

Yeast protein extracts made according to the above description were mixed with labeled oligonucleotides for 20 min at 30°C, then loaded onto 5% PAGE gels for DNA protein separation. The PAGE gels were dried and put into a film cassette for autoradiography.

GST-pulldown

Human flotillin-1 is 98% identical to the mouse protein and has a 47% identity with human flotillin-2 (Edgar and Polak, 2001). The pGEX-KT mouse plasmid containing full length mouse flotillin-1 was transformed into the DH5α strain of E. coli. A 4 ml overnight culture was inoculated into 200 ml LB media and shaked at 200 rpm in 37°C for 120 min. IPTG was added to the culture at 0.5 mM for 4 h to induce flotillin-1 expression.
E. coli. were harvested and washed once in ice cold STE buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA). E. coli. were suspended in 8 ml STE buffer containing 5 mM DTT, 40 µM leupeptin, 20 µM pepstatin, and 1 mM PMSF. N-lauryl-sarcosine was added to a final concentration of 1.5%. The E. coli were vortexed and then sonicated (output = 1, cycle = 10%, 45 sec in ice). The resulting lysate was centrifuged at 15,000 x g for 10 min to obtain a clear supernatant. Triton X-100 was added to 2%.

The supernatant was incubated with Glutathione-Agarose (1 ml/200 ml E. coli. culture lysate) for 60 min at 4°C. The gel slurry was washed with PBS buffer containing 1% Triton X-100 6x5 min, and stored in PBS.

As a negative control of GST-flotillin pulldown, GST-flotillin glutathione agarose beads were washed with PBS several times to remove Triton X-100. Ten units of thrombin was added to the bead slurry for cleavage at room temperature for 4 h. Cleaved flotillin-1 was washed from the gel with 5 ml 1% Triton X-100 PBS buffer, 6x10 min. The efficiency of thrombin cleavage to GST-flotillin protein was detected by Western blot using anti-flotillin antibody. No intact GST-flotillin-1 fusion protein was detected on the gel.

A HEK293 cell line expressing calpain10 DIIIX2 under the control of the pTRE promoter was induced by addition of 1 µM doxycycline to the culture medium for 96 h. Cells were washed three times with cold HBSS. Five dishes of cells were scraped and lysed in 5 ml lysis buffer (40 mM Tris, 150 mM NaCl, 2 mM EDTA, 1 mM DTT, 50 iM leupeptin, 4 iM pepstatin, 50 iM PMSF, 0.5% NP-40, 0.1% deoxycholate, pH 7.4). The lysate was passed through a 25-gauge needle for better lysis, and subjected to centrifugation at 7,000 g for 10 min. The lysate was diluted 7-fold in lysis buffer without
detergents, and incubated with 20 µl of GST-flotillin agarose beads for 2 h at 4°C. The beads were sequentially washed 5 min with buffer (pH 7.4 40 mM Tris, 150 mM NaCl, 2 mM EDTA, 1 mM DTT) containing 0.1%, 0.2%, 0.3%, 0.4%, 0.5% NP-40. The beads were finally washed briefly with PBS buffer to eliminate detergent before boiling in 40 µl SPB buffer for SDS-PAGE and western blot assay.

**Yeast transformation**

Yeast strains was streaked on YPDA plates to form colonies. Individual colonies were picked and grown in YPDA media until O.D.550 reached 0.4-0.6. Yeast cultures were harvested and washed in H2O once, then resuspended in 1xTE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) containing 100 mM LiAc. Yeast cells were centrifuged, and resuspended in 1xTE+LiAc buffer. For every 20 ml yeast culture, the final suspension volume was 500 µL. A 50 µL aliquot of suspension was then mixed with 50 µg ssDNA and 200 ng plasmid. An aliquot of 300 µl transformation solution containing 1xTE+LiAc+40% PEG4800 was mixed with the yeast suspension and shaken at 200 rpm at 30 °C. DMSO was added to the mixture to form a final 10% concentration, and the yeast were heat shocked at 42°C for 15 min with occasional shaking.

Supernatant was removed after yeast cells by centrifugation at 1,200 g for 3 min at room temperature. The yeast pellet was resuspended in 1xTE buffer, then spreaded onto SD agar selection plates.
RESULTS

Identification of a calpain-like protein (Cpl) in *Dictyostelium discoideum*. A search of the *Dictyostelium* DNA databases available on the internet revealed two EST clones (SLC473 and SSC516) at the Tsukuba *Dictyostelium* cDNA project that appeared to encode structures containing calpain D-III domains. The largest genomic DNA sequence including both SLC473 and SSC516 was *Dictyostelium* contig 13130 (Sanger Institute *Dictyostelium* sequence database), which contained a three exon gene, predicted by the fgenesh program (Salamov, Solovyev, 1999, unpublished data, CGG WEB server: http://genomic.sanger.ac.uk/gf/gf.html ) when using the yeast gene setting, and allowing GC splicing. The predicted 646 residue protein would have a molecular mass of 72,278, and contain tandem domains homologous to DIII from mammalian calpains (Fig. 3). Blastp analysis indicated closest homology to mouse calpain-10 (E score = 2 X 10^{-10}).

After obtaining the full-length cDNA, we found that Cpl resembles calpain-10 in the DIII domains and the Cpl knockout strain had a phenotype similar to that obtained by ablation of calpain function in mammalian cells (E. Czerwinski and R.L. Mellgren, unpublished data). These similar phenotypes included aspects such as cell motility, adhesion, and detachment.

It was impossible to pinpoint the catalytic cysteine residue of Cpl based on sequence comparison (Fig. 4) between the calpain and Cpl. To prove Cpl is a protease like calpain, it is imperative to obtain 100% purity of native Cpl for proteolysis and autoproteolysis assay. The expression of Cpl in a fusion-cleavage expression system such as GST-thrombin system could resolve this puzzle in future studies.
A. Gene structure predicted by the fgenesh program. *Dictyostelium* contig 13130 was analyzed, and a single 3-exon gene was predicted.

B. Predicted amino acid sequence of Cpl. After purification of Cpl from phenyl-sepharose, the N-terminal sequence was confirmed by direct protein sequencing (bold underlining). A 50 kDa autoproteolysis fragment was found from cleavage after Pro 189 (double underlining). Putative active site His and Gln sites are boxed in. Conserved sequence motifs of the two calpain D-III domains are represented in bold type. The entire cDNA sequence was confirmed by reverse transcriptase-PCR of the Cpl mRNA and DNA sequencing.

C. Comparison of the putative active site His and Gln residues of Cpl with the calpain consensus sequence, and the sequence in human calpain-10.
A

B

C

Putative active site His and Gln residues in Cpl:

Consensus: HAY (17 a.a.) LRLRNPWG (80-86 a.a.) Domain III
Calpain 10: HAF (18 a.a.) LRIQNPWG (86 a.a.) Domain III
Cpl: HWI (17 a.a.) LKLQPEG (110 a.a.) Domain III
Calpain 10 contains a Cys-His-Asn catalytic triad in domain II, but there is only a plausible catalytic triad found in Cpl based on homology comparison. Sequence NPKF/NPCF in calpain-10 and NPQY/NPQF in Cpl are motifs that appear to be involved in membrane vesicle transport (Mishra et al., 2002).
Since no one has studied the Cpl protein and gene before, Northern blots were carried out to obtain information on Cpl expression. There was a single 2.3Kb transcripts of Cpl (Fig. 5). Under vegetative shaking culture conditions, there was no alternative splicing variant found. There was no alternative splicing variant in Dictyostelium slugs either. We do not know whether Cpl in Dictyostelium will have other isoforms when cultured under different conditions.

Purification and Characterization of Cpl

Protein immunoblot analysis using anti-Cpl antibody, indicated that Cpl can be easily extracted with neutral buffers from glass-bead ruptured cells (data not shown), indicating that it is a soluble protein. Gel filtration and sucrose density gradient ultracentrifugation of Dictyostelium cell lysates indicated that the native Cpl was an asymmetric monomer with a calculated molecular mass of 78,900 Daltons and a frictional coefficient of 1.46 (Table II). DEAE-Sepharose chromatography of Dictyostelium amoebae lysates demonstrated co-migration of Ca$^{2+}$-activated caseinolytic activity with Cpl immunoreactivity, both eluting from the column at about 250 mM NaCl in a linear salt gradient.

These preliminary studies allowed us to devise a purification scheme for Cpl comprising sequential chromatographies on DEAE-Sepharose, Bio-Gel A1.5m, and Phenyl-Sepharose as described in the Materials and Methods section. The caseinolytic activity associated with Cpl immunoreactivity was sensitive to calpain inhibitors, but not to pepstatin A or PMSF, inhibitors of aspartyl and serine proteases, respectively (Table III). Chromatography on Phenyl-Sepharose was highly effective in separating Cpl from contaminating proteins (Fig. 10A). The final purified product had a single major protein
Fig. 5. Cpl mRNA Expression in *Dictyostelium*

Northern blot analysis of total RNA from *Dictyostelium* amoebae or slugs revealed a single RNA species at approximately 2.4 kb by utilizing probes from either of the ESTs obtained from the Tsukuba *Dictyostelium* EST project (Fig., middle panel, SLC473 and SSC516). Although there appeared to be less Cpl mRNA in slugs, there was also much less beta-actin detected (Fig. 5, right panel). The total RNA preparation appeared intact as the ribosomal RNA bands were intact in both the amoebae and slug RNA samples (Fig. 5, left panel).
S = RNA standards; A = amoebae RNA; SL = slug RNA
band at 77 kDa, which co-migrated with the Cpl immunoreactivity (Fig. 10B). Trace amounts of 50 kDa protein also were present in this preparation. In several other preparations of Cpl, both the 60 kDa and 50 kDa bands were observed.

Dictyostelium strain AX3 was cultured in HL5 media (17.8 g Oxoid peptone, 7.15 g yeast extract, 0.54 g Na₂HPO₄, 0.4 g KH₂PO₄, 2% glucose, Vitamin B12 5 µg, folate 200 µg per liter). Media was autoclave twice for 20 min and cooled overnight between autoclave cycles. Glucose was added to media till final 2% and Fungi-Bact antibiotics also was added before use.) and grown till 4-6x10⁶ cells/ml before inoculating into SM broth E. coli. media for massive culture (E. coli. was harvested after cultured in LB till saturated. E. coli. pellet was resuspended in SM broth media. Media was autoclave twice for 20 min and cooled overnight between autoclave cycles. Glucose was added to media till final 1% before culture. SM broth media: 10 g Oxoid peptone, 1 g Oxoid yeast extract, 1 g MgSO₄, 1.9 g KH₂PO₄, 0.6 g K₂HPO₄ per liter pH 6.0 to 6.4). The optimal Dictyostelium density for harvest is <6x10⁶ cells/ml. Over-culture resulted in loss of the native band, and Cpl immunoreactivity was present as degraded fragments.

Batch DEAE-Sepharose Chromatography of Cpl. Dictyostelium was harvested and lysed as described in the Methods section. The crude extraction was applied to a DEAE column equilibrated in pH 6.4 50 mM Imidazole plus 50 mM NaCl buffer, then the procedure described in the Methods section was followed. Cpl-containing fractions were detected by Western blot Fractions were detected by Western blot analysis using the Cpl antibody.
Fig. 6. **DEAE-Sepharose Chromatography of Cpl Detected by Western blot**

The 12,000 x g supernatant from $10^{10}$ Dictyostelium cells was applied to DEAE-chromatography as described in the Methods section. Cpl was eluted from the gel by increasing the buffer NaCl concentration to 0.32 M and 0.5 ml fractions were collected. The majority of Cpl immunoreactivity in Western blots appeared between fractions 2 and 9.
Fig. 7A. Bio-Gel-A1.5m Chromatography of Cpl

The Cpl protein peaked at tube #46 of the Bio-Gel column, corresponding to a Stokes radius of 4.4 nm. Fractions were analyzed by Western blotting using anti Cpl antibody. The numbers under the blot are gel filtration fraction numbers.
Fig. 7B. Protease Activity and Western Blot of Gel Filtration Fractions

Bio-Gel-A1.5m gel filtration chromatography of another Cpl preparation. Because larger volume fractions were collected in this experiment, Cpl protein peaked at tube #34. The numbers under the blot are gel filtration fraction numbers. The graph presents a protease activity assay from this experiment. The immunoreactivity peak matched with the first proteolytic activity peak.
Bio-Gel A-1.5m Chromatography of Cpl. The fractions (8-16 ml) containing Cpl were pooled and concentrated to less than 3 ml by dehydrating against dry polyethylene glycol (cut off MW = 3,000) in a dialysis tube. The concentrated sample was applied to an 80 ml Bio-Gel A-1.5m gel filtration column pre-equilibrated with buffer containing protease inhibitors (50 mM Imidazole, 100 mM NaCl, 1 mM EGTA, 1 mM DTT, 10 μM leupeptin, 5 μM pepstatin, 0.1 mM PMSF, pH 6.4). The flow rate was approximately 4 to 6 ml per h, and 1.6 ml fractions were collected.

Prior to assay, chromatography fractions were centrifuged in Centricon YM-30 tubes to remove protease inhibitors, and resuspended in an equal volume of buffer (pH 6.4, 50 mM Imidazole, 100 mM NaCl 1 mM DTT) without protease inhibitors. Calcium-activated caseinolytic activity was determined on chromatography fractions by using $^{14}$C-methylated casein (1000 cpm/μg) as the substrate (Mellgren et al., 1982). Briefly, fractions were incubated with or without 5 mM calcium acetate at 25°C with 0.5 mg/ml $^{14}$C-casein. After 1-4 h, TCA was added to 2.5%, and the samples were centrifuged to remove precipitated casein. Radioactivity in the supernatant fraction was counted in a liquid scintillation counter to determine proteolytic release of TCA-soluble peptides from casein. Ca$^{2+}$-activated caseinolysis was determined by subtraction of activity measured in the absence of calcium from activity measured in its presence. A unit of protease activity generates 1ng of acid-soluble casein fragments per min under the standard assay conditions (Mellgren et al., 1982).
Fig. 8. Cpl Assay after DEAE Gradient Elution

The amoebae lysate was applied to a DEAE-Sepharose column, and Cpl was eluted with a linear NaCl gradient as described in the Methods section. Fractions were analyzed for Cpl immunoreactivity (filled circles), calcium-activated caseinolysis (hollow circles), and NaCl concentration (x).
Sucrose gradient ultracentrifugation of Cpl. An AX-3 lysate was spun at 10,000xg for 10 min and loaded onto the top layer of a sucrose density gradient tube. The 4 ml sucrose gradient volume was aliquoted into 19 fractions after 15 h centrifugation at 150,000xg. Western blot of those fractions is shown below (Fig. 9).

**Autoproteolysis of Cpl**

Phenyl-Sepharose purified Cpl had no detectable caseinolytic activity when assayed. However, when the preparation was concentrated and pre-incubated in the absence of protease inhibitors for several hours, the 77 kDa Cpl band was converted to several 40 - 50 KDa immunoreactive forms, and caseinolytic activity was generated. These results suggest that autoproteolysis of the 77 kDa form increases Cpl proteolytic activity, and is reminiscent of the autoproteolysis of mammalian calpains. To investigate whether the proteolysis was caused by a contaminant in the Phenyl-Sepharose fractions, individual Cpl tubes around peak fractions from the Phenyl-Sepharose chromatography were incubated in the absence of inhibitors. The rate of autoproteolysis was estimated by comparison of immunostaining densities of the 77 kDa, 60 kDa, and 50 kDa bands. The rate of generation of proteolysis products was essentially the same over the entire Phenyl-Sepharose Cpl peak. This result strongly suggests that Cpl undergoes autoproteolysis as a contaminating protease would probably migrate with a different mobility upon gradient elution from Phenyl-Sepharose, thereby producing preference for proteolysis at one or the other ends of the Cpl peak. Autoproteolysis of Cpl was inhibited by leupeptin and EGTA, confirming the presence of a calpain-like protease activity.
Fig. 9. Western Blot of Sucrose Gradient Fractions

The Cpl peaked at fraction #7 (lane 11). Two protein standards: myoglobin with $S_{20,w}=2$ and catalase with $S_{20,w}=11.3$ peaked at tube #4 and #15, respectively. Cpl had an $S_{20,w}=4.3$ based on its migration relative to the protein standards.

- lane 1: lysis buffer only
- lane 2: Ax3 strain lysate
- lane 3: pellet after lysis
- lane 4: blank
- lane 5-15: sucrose gradient ultracentrifuge fractions 1-11
<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stokes radius</td>
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</tr>
<tr>
<td>S20,w</td>
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</tr>
<tr>
<td>Partial specific volume</td>
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<tr>
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<tr>
<td>f/f₀</td>
<td>1.46</td>
</tr>
<tr>
<td>Mass by SDS-PAGE</td>
<td>77,000 Daltons</td>
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<tr>
<td>Mass predicted from cDNA sequence</td>
<td>72,278 Daltons</td>
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</table>
Table III: Inhibition of Cpl-associated Caseinolytic Activity by Protease Inhibitors

Cpl purified through the Bio-Gel chromatography step was assayed for Ca$^{2+}$-dependent caseinolytic activity as described in the Experimental Procedures section, in the presence of various protease inhibitors.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration, $\mu$M</th>
<th>Percent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylmethane-sulfonyl fluoride</td>
<td>500</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>100</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>50</td>
<td>82</td>
</tr>
<tr>
<td>Calpeptin</td>
<td>10</td>
<td>80</td>
</tr>
</tbody>
</table>
Fig. 10. Phenyl-Sepharose Chromatography of Cpl

Pooled 77 kDa Cpl fractions from the Bio-Gel purification step were applied to a Phenyl-Sepharose column and eluted with a decreasing NaCl gradient as described in the Materials and Methods section. Panel A: 77 kDa immunoblot relative density (filled circles), protein concentration (open circles), and NaCl concentration (---) were determined. After application of the NaCl gradient, tightly bound proteins were eluted with buffer A containing 1% NP-40 detergent (arrow). Panel B: Pooled aliquots of fractions 3 through 9 from the Phenyl-Sepharose chromatography were concentrated approximately 20-fold by Centricon filtration. Approximately 200 ng concentrated samples were subjected to electrophoresis and coomassie blue staining (CB), or immunoblot analysis for Cpl (IB).
Fig. 11 Western Blot of Final Purification of Cpl Protein

A polyacrylamide gel containing the 75 kDa and 50 kDa Cpl protein bands was transferred to PVDF membrane and the bands were micro-sequenced. The Cpl N-terminal sequence was obtained from the 75 kDa band and it matched the predicted sequence from the cDNA. One internal cleavage site was identified from the 50 kDa band sequence.
Phenyl-Sepharose purified fractions of Cpl were concentrated as described in the Experimental Procedures section. Panel A: Concentrated fraction 9 from the experiment depicted in Fig. 8 was analyzed by SDS-PAGE and coomassie blue staining (CB) or immunoblotting for Cpl (IB). Panel B: Phenyl-Sepharose fractions representing the Cpl peak were concentrated separately, and relative density of the 40-50 kDa Cpl immunoreactivity in immunoblots was determined (hollow circles). Caseinolytic activity in the absence of calcium also was measured (filled circles).
Panel A: Phenyl-Sepharose fractions were concentrated and incubated without protease inhibitors for 2.5 h at 23°C, then subjected to immunoblot analysis for Cpl. A different Cpl preparation than the one presented in Fig. 6 was used, and it contained somewhat more 60 kDa and 50 kDa fragments (panel A, \( t_0 \) fractions). Because the Phenyl-Sepharose column was prewashed with more 2 M NaCl buffer in this experiment, Cpl appeared later in the fraction collector. However, Cpl still eluted at 1 M NaCl in the gradient.

Panel B: Percent proteolysis of the 77 kDa Cpl versus time of incubation in the absence of inhibitors. Concentrated Phenyl-Sepharose fractions containing purified Cpl were incubated in the absence of protease inhibitors for 2.5 hrs at 23°C, or for 72 h at 4°C. Percent proteolysis of the 77 kDa Cpl was determined by densitometric scans of the resulting immunoblots. It was calculated as the sums of the densities of the 60 kDa and 50 kDa bands, divided by the total staining intensity for all Cpl bands, and multiplied by 100.
Fig. 14. Leupeptin and EGTA Protect against Autoproteolysis of Purified Cpl

Pooled concentrated Phenyl-Sepharose purified Cpl was incubated at 23°C for 4 h in the absence of protease inhibitors (0). Other samples included the inhibitor cocktail used in the isolation buffers (all), 50 μM leupeptin (leu) or 5 mM EGTA (E).
To find out the N-terminal sequence of Cpl, and to identify the cleavage site for the major degradation form, samples of native 77 KDa Cpl and 50 KDa fragment were submitted for amino acid analysis to the Emory University Microchemical facility. The N-terminus sequence was TESPTTTTTTTT predicted by the fgenesh program. The N-terminus of the 50 kDa fragment was AKKVKA, a cleavage occurring after proline 189, to produce a 51,291 Dalton major fragment.

**Phylogenetic Analysis of the Cpl DIII Domains**

Homology searches utilizing the cDNA sequence for Cpl revealed the presence of genes encoding D-III domains in several protists, including the rhizopod, *Entamoeba histolytica* (*E. histolytica*). The Sanger Institute *E. histolytica* contig 4596 includes a single open reading frame encoding a putative 591 residue protein that we have provisionally named Eh Cpl, containing three consecutive D-III domains. Several TIGR *E. histolytica* EST clones were found to correspond to parts of the coding sequence in contig 4596, suggesting that this gene is expressed. A cladistic analysis reveals several early branches containing protist D-III domains and the Cpl D-III domains (see Fig. 16). Among human calpains, Capn 7 and Capn 10 D-III domains are most closely related to the Cpl and protist sequences. More distantly related to the protist D-III domains in the plant DEK1-like, Capn 5, and other metazoan calpains.
The D-III domains were analyzed for relatedness using the Clustalw program. When D-III domains are present more than once in a sequence, they are lettered a, b, etc., in order starting from the amino terminus. Eh = *Entamoeba histolytica*; TbCh6 = *Trypanosoma brucei* calpain on chromosome 6; Dd = *Dictyostelium discoideum*; Yl = *Yarrowia lipolytica*; Hs = *Homo sapiens*; An = *Aspergillus nidulans*; Pf = *Plasmodium falciparum*; Dm = *Drosophila melanogaster*; At = *Arabidopsis thaliana*; Zm = *Zea mays*; Ce = *Caenorhabditis elegans*. Abbreviations following the organism name are accepted names for the calpains identified. Numbers following organism names are accession numbers for the nucleotide sequence.
Fig. 16. Cpl Binding to Acidic Phospholipids

Two mg of PI, PS, PE, PC phospholipids was dissolved in chloroform, then dried in nitrogen gas flow until all lipids were evenly distributed on the bottom of glass tubes. Membrane vesicles, made by sonicated 20x4 sec after 500 µl buffer (50 mM Imidazole, 150 mM NaCl, pH 6.4) were added to the tube. Cpl from DEAE-batch chromatography was incubated with the membrane vesicles for 5 min at room temperature, then loaded onto a Sepharose-2B column for size separation. Cpl was detected by Western blotting. PI = phosphatidylcholine; PS = phosphatidylserine; PE = phosphatidylethanolamine; PC = phosphatidylcholine.
Cpl binds to acidic phospholipids

PL ↑ Cpl ↓

PI
PS
PE
PC

2 3 4 5 6 7 8 9 10 11 12

fraction number
Using the yeast-two hybrid system to identify calpain-10 binding partners. cDNA fragments of Cpl and calpain-10 containing DIIIX2 domains, around 340 amino acids in length, were PCR amplified and subcloned into pGBKT7 vector. PCR was run for 10 cycles with pfu polymerase under the following cycle conditions: 94°C 45 sec, 94°C 45 sec + 50°C 45 sec + 72°C 2 min. For the last cycle, elongation was at 72°C for 10 min. Several yeast clones containing the pGBKT7 vector with tandem DIII domain cDNA insert were cultured and analyzed by Western blot using anti-myc antibody. The blots are indicated in Figs. 17 and 18.

Since there are many calpain family members containing DIII domain (see Literature section), we decided to check the specificity of interaction between preys and different calpains DIII domains. DIII domains from different calpain were PCR engineered and subcloned into pGBKT7 vector with NcoI and SalI cuts. Plasmids with different BD-DIII bait were transformed into yeast strain AH109. The expression of BD fusion protein in yeast AH109 can be detected by Western blot analysis using anti-myc 9E10 antibody as there was a myc epitope between the BD domain and fusion protein. After Western blot verification, yeast strains containing different baits were mated with positive preys for interaction assay. Calpain species used in this specificity assay were: human calpain 1, rat calpain 2, human calpain 3, mouse calpain 5, human calpain 6, and human calpain-10. All were expressed at the expected size (Fig. 19).
Fig. 17. Western Blot of BD-Cpl-DIIIX2 Expression in Yeast AH109 Detected by Mouse anti Myc Antibody

lane 1: myc antigen positive control          lane 2: protein marker
lane 3: yeast AH109 strain                  lane 4: AH109 strain with pGBK7 plasmid
lane 5: AH109 containing BD-Cpl-DIIIX2 clone 1a
lane 6: AH109 containing BD-Cpl-DIIIX2 clone 1b
lane 7: AH109 containing BD-Cpl-DIIIX2 clone 3a
lane 8: AH109 containing BD-Cpl-DIIIX2 clone 3b
lane 9: AH109 containing BD-Cpl-DIIIX2 clone 3c
Fig. 18. Western Blot of BD-Capn10-DIIIX2 in Yeast AH109 Detected by Mouse anti Myc Antibody

Only yeast expression capn10DIIIX2 bait can be detected
lane 1: protein marker
lane 2: yeast AH109 strain
lane 3: AH109 strain with pGBK7 plasmid
lane 4: AH109 containing BD-Capn10-DIIIX2 clone 1, 2 d culture
lane 5: AH109 containing BD-Capn10-DIIIX2 clone 1, log phase culture
lane 6: AH109 containing BD-Capn10-DIIIX2 clone 2, log phase culture
lane 7: AH109 containing BD-Capn10-DIIIX2 clone 3, log phase culture
Yeast clones containing different baits were cultured until late log phase. Yeast protein was extracted by glass beads disruption, then subjected to western blot analysis. Although little capn6 DIII protein was observed in this experiment (lane 11), in other experiments it was present at the predicted size.
cDNA containing DIIIX2 domains of calpain-10 and flanked by NcoI and PstI cleavage sites was subcloned into the pGBKT7 bait vector. The plasmid was transformed into yeast strain AH109. Visible clones formed on SD-Trp-His plates indicating no endogenous transcriptional activity by this bait alone.

The bait yeast AH109 clone containing pGBKT7+calpain10 DIIIX2 in SD-Trp media was grown overnight until the cell number was $5 \times 10^9$. Bait yeast of $1 \times 10^9$ cells was mated with 1ml Clontech human liver library cells ($5 \times 10^7$ cells/ml in yeast Y187 strains) according to the Clontech technical manual.

The mating mixture was spread onto SD-Leu-Trp-His (low-stringency) plates for the yeast two hybrid screen.

Prey plasmid was retrieved from representative clones of the redundant positive yeast clones, and self-activation of reporter genes without bait was examined. Only prey clone 24 was found able to trigger the expression of report gene without the presence of bait protein, and it was thus regarded as a false positive clone.

Yeast AH109 strains with baits containing different DIII domains from calpain family members were mated with yeast Y187 strains with prey clones 1, 3, 7, 19, 27, 31. The latter are representative of all of the redundant prey clones identified.

The mating mixtures were streaked onto SD-Leu-Trp plates for mating quality verification, onto SD-Leu-Trp-His plates for intermediate stringency selection, and onto SD-Leu-Trp-His-Ade plates for high stringency selection. As shown in Table IV, all the matings were successful. Some baits containing the DIII domains from different calpain members other than from calpain-10 can interact with prey weakly and sporadic clones can be found on SD-Leu-Trp-His plates. However, DIIIX2 from calpain10 always
Table IV. Yeast Baits Containing DIII Domains from Different Calpains Mated with 6
Positive Preys Obtained from Two-hybrid Screening

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<th></th>
<th>C10 DIII</th>
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SD-2: SD-Leu-Trp
SD-3: SD-Leu-Trp-His
SD-4: SD-Leu-Trp-Ade-His

+ means several clones
+a means 1/5 of +++
++d means 1/2 of +++
+b means 1/10 of +++
++ means 1/2 of +++
prey 7: IBtK; prey 19: α -HS-glycoprotein; prey 27: flotillin 1
presented the strongest interaction. DIIIb interacted with prey weakly. Although the DIIIa domain from calpain-10 seems to account for the major interaction with prey, by itself it is not sufficient for strong interaction with prey. The combination of DIIIa and DIIIb domain is required for full interaction. This confirmed our beginning hypothesis that it is the tandem DIIIX2, not individual DIII domains, that have an important targeting function in calpain-10 (Fig. 20).

Flotillin-1, clone 27, was identified as a positive prey interacting with calpain10 DIIIX2. Flotillin was first cloned as a protein homolog to epidermal surface antigen (ESA) (Bickel et al., 1997). The highly conserved Cys34 residue in flotillin PHB domain is required for membrane targeting (Isabel et al., 2002). The N-terminus of flotillin contains a prohibitin-like domain (PHB) homologous to a number of proteins associated with raft domains including stomatin, podocin, and prohibitin (Morrow et al., 2002).

Flotillin can co-immunoprecipitate with the CAP and cbl, insulin receptor substrates. Overexpression of CAP without SH3 domain can attenuate the insulin induced glucose metabolic effects (Baumann et al., 2000) and believed to be one of the pathway along which insulin signals is transmitted to GLUT4-bearing membrane in the cell (Czech, 2000). Inside CAP a 115 amino acid region homologous to sorbin is responsible for flotillin binding (Kimura et al., 2001).

After Y2H screen, it is natural to find out which part of the flotillin is responsible to the interaction with calpain10 DIIIX2. We arbitrarily tried three fragments of mouse flotillin-1. The PHB domain of flotillin appears to be required for the interaction with calpain-10 DIIIX2 domains (Fig. 21).
Fig. 20. Mating Assay of Yeast Containing DIII Domain from Different Calpain Members Mated with 6 Positive Clones Obtained from Two-hybrid Screen under Different Selection Stringency

Example of prey proteins specific interaction with different baits including BD-calpain10 DIIIX2 on different stringent selection plates.

Baits investigated for mating assay:
BD-capn10 DIIIX2 (positive) BD-Lamin C (negative control)
BD-Cpl DIIIX2 BD-p53 (negative control)
BD-capn1 DIII BD-capn2 DIII
BD-capn3 DIII BD-capn5 DIII
DIII BD-capn6 DIII BD-capn10 DIIIa
BD-capn10 DIIIb
Prey constructs were designed, expressing the N-terminal, C-terminal and central regions of mouse flotillin-1. These yeast bearing vectors were mated with yeast expressing capn10DIIIX2 from the bait vector. Colonies were detected on SD-Leu-Trp-His selection plates.

Prey 1: The N-terminal 1/2 peptide of mouse flotillin-1 (amino acid residues 14-232)

Prey 2: The middle 1/2 of mouse flotillin-1 (amino acid residues 140-278) fused with BD domain in pGADT7 as a prey

Prey 3: The C-terminal 1/2 peptide of mouse flotillin-1 (amino acid residues 215-427) fused with BD domain in pGADT7 as a prey
DISCUSSION

*Dictyostelium discoideum* expresses a novel protein named Cpl, for calpain-like protein. Cpl homology to mammalian calpains exists mainly within its tandem D-III domains. It is important to note that until now D-III domains had only been found in calpains. The amino terminal sequence of Cpl does not resemble calpains, except for the presence of correctly positioned His and Gln charge-transfer residues within the putative catalytic domain. The catalytic Cys cannot be determined by homology comparison with known calpains. It would have to be different from the calpain DCW consensus sequence, which does not appear in Cpl. Nevertheless, we were able to show that a weak cysteine protease activity is associated with Cpl during purification, detectable by cleavage of exogenous substrate (casein) or by Cpl autoproteolysis.

Identification of the physiologic function of Cpl is of interest, especially since its global structure resembles that of calpain-10 which is a putative diabetes-related protein. Cpl has much weaker caseinolytic activity when compared with the conventional calpains. Several different preparations of purified human erythrocyte μ-calpain had turnover numbers of 200 to 400 μg casein/min/nMol calpain under standard assay conditions (data not shown). By contrast, the concentrated, activated Phenyl-Sepharose Cpl activity had an estimated turnover number approximately 1000-fold lower than calpain 1. The turnover number of Cpl may be underestimated because we have not yet optimized conditions for its autolytic activation. However, it is doubtful that it will achieve the caseinolytic potential of μ- or m-calpains. Unlike the conventional calpains, Cpl might have different substrate specificity, and is thus unable to efficiently cleave
model substrates like casein. Because Cpl does not have a calpain-like catalytic domain based on primary sequence homolog comparison, it is impossible to decide if Cpl does function as a protease in *Dictyostelium*. Further studies will be required to address these issues. A suitable resolution may be to express GST-Cpl in Dictyostelium, and purify it by affinity chromatography. Cpl close to 100% purity generated from the GST fusion protein by thrombin cleavage will be a good product for proteolysis and autoproteolysis assays.

The general structural and functional properties of Cpl suggest that it is distantly related to the calpain family. The recently described calpain-like proteins in the protist *Trypanosoma brucei*, TbCALP1 and TbCALP2 (Hertz-Fowler et al., 2001), appear to be substantially different from either Cpl or calpains. Unlike Cpl, their homology to calpains is strictly associated with the catalytic region. However, because neither TbCALP1 nor TbCALP2 appear to possess active site Cys or His residues (Hertz-Fowler et al., 2001), it seems unlikely that they have calpain-like protease active sites. The question of their potential proteolytic activity awaits characterization of the expressed proteins.

Phylogenetic comparison of the D-III domains including the new information on protist and *Dictyostelium* sequences extends the recent study by Sorimachi and Suzuki. In general, the results agree, except that the present analysis places Capn 7 and Capn 10-like D-III domains closer to the root of the tree. In fact the first several branches contain only the protist or myxamoeba D-III with the exception of one Capn 7 ortholog, *Y. lipolytica* PaLB. The phylogenetic analysis also shows that the two human Capn 10 D-III domains are more similar to each other than to any of the multiple D-III domains in Cpl or Eh Cpl. It is, therefore, likely that a more recent duplication resulted in the tandem D-III domains.
present in Capn 10. The occurrence of D-III duplication more than once during evolution suggests that this event produces an important alteration or enhancement of D-III function in some organisms.

**Proteolytic Activity of Cpl.**

It was not possible to pinpoint the catalytic cysteine residue by homologous primary sequence comparison between the calpain and Cpl. To prove Cpl is a protease like calpain, it will be necessary to obtain native, highly purified Cpl for proteolytic activity assay. The expression of Cpl in a fusion-cleavage expression system such as GST-thrombin system may resolve this aim in future studies.

**Yeast Two-hybrid Screen Results and Positive Clones Analysis**

We obtained 70 positive clones using BD-calpain-10 DIIIx2 as bait for interaction from Y2H screening by BD-calpain10 DIIIx2 domain as bait versus a fetal human liver library. DNA sequence of these positive clones revealed several interesting cDNAs. The following is the important prey list after the Y2H screen, but before further co-IP confirmation:

- **Clone 1:** cDNA coding for a protein with presently unknown function.
- **Clone 3:** Homo sapiens S-phase kinase-associated protein 1A (p19A) (SKP1A), transcript variant 2.
- **Clone 7:** Homo sapiens inhibitor of Bruton's tyrosine kinase (Ibtk), involved in lymphocyte survival.
- Clone 19: Human alpha-2-HS-glycoprotein. A putative inhibitor of insulin receptor tyrosine kinase (Arnaud and Kalaby, 2002; Cintron et al., 2001). It also significantly augmented phagocytosis of apoptotic cells by macrophages, and labeled dextran 70,000 during macropinocytosis (Jersmann et al., 2003).

- Clone 27: Homo sapiens flotillin-1: a lipid raft protein involved in membrane vesicle transport. The formation of a ternary complex of CAP, cbl and flotillin-1 plays an important function in insulin-induced glucose transport (Baumann et al., 2000).

- Clone 31: Homo sapiens calsyntenin 3 (CLSTN3), a transmembrane protein containing a highly acidic cytoplasmic segment with putative Ca(2+)-binding regions. It may be a false positive clone as the fusion part is in the 3-UTR non coding region.

- Clone 47: alpha-2-macroglobulin.

Clones 3, 7, 19, and 27 are inserted in the correct reading frame. Because of the proposed role of calpain-10 in insulin signaling, we focused on clone 27, flotillin-1. The possibility of a false positive interaction was excluded by mating with BD-Lamin C and BD-p53 negative control, and further by Y2H experiments with DIII domains from different calpain members.

Flotillin-1 has been described as a lipid raft component associated with caveolae in neurons (Edgar and Polak, 2001) and was patchy and prominent on the plasma membranes of synapses in the neurophil (Kokubo et al., 2003).
CONCLUSIONS

1. Cpl containing tandem DIII domains is homologous to calpain family members and closest to calpain10.

2. The protein shape of Cpl seems elongated given the information from gel filtration and sucrose gradient ultracentrifuge.

3. Full-length Cpl cDNA was cloned by RT-PCR after the peptide microsequence information on the highly purified major 75-80KDa protein band.

4. Cpl may undergo limited autoproteolysis before its proteolytic activation. This issue can be finally resolved if 100% purity Cpl protein were obtained after GST-Cpl protein expressed in Dictyostelium, purified by Glutathione-agarose and cleaved from agarose by thrombin.

5. Several positive preys obtained clones after Y2H screen could provide some clues toward understanding the function of DIII domains, and their possible role in calpain-10 function. These preys interact specifically with the DIII domain from calpain-10 instead of the other calpains. Pulldown assay and cellular function studies may provide more information about calpain-10 interactions.

6. Flotillin-1 seems to be an important mediator in the insulin-induced glucose transportation pathway between calpain-10 and CAP/cbl, the insulin receptor substrates. However, further cellular studies of calpain-10 into this pathway are needed for clarification.
SUMMARY

Calpains are intracellular, cysteine proteases found in mammalian cells and even in some lower organisms (Sorimachi et al., 1997; Delaney et al., 1991). There is evidence that calpain 1 and calpain 2 are important mediators in various cell procedures including adhesion and motility in animal cells (Shiraha et al., 1999).

Calpain 3, also named p94, is mainly expressed in skeletal muscle tissue (Federici et al., 1999; Herasse et al., 1999), and loss of its proteolytic activity by genetic mutation is the cause of human disease limb girdle muscular dystrophy type 2A (LGMD2A) (Richard et al., 1995; Ono et al., 1998). From studies of linkage analysis and single nucleotide polymorphisms in the non-coding region of calpain-10 gene in several population (Horikawa et al., 2000), calpain-10 was regarded as a factor associated with type 2 diabetes. Whether the different splicing variants (Horikawa et al., 2000) represent a regulation mechanism is still clear. In general, studies on the role of calpain-10 to type 2 diabetes is less clear than of calpain 3 to LGMD2A.

Because the cellular slime mold, *Dictyostelium discoideum*, is a genetically tractable model for cellular studies (Kessin, 2001). In this organism we have investigated a calpain-10-like protein which may provide useful clues towards an understanding of the function of calpain-10. Contig 13130 (Sanger Institute *Dictyostelium* sequencing project) was identified as a 3-exon gene that encodes a calpain-10-like protein later identified in our lab as a 72,278 Da cytosolic protein. Cpl domain structure resembles mammalian calpain-10 with a plausible N terminal catalytic domain followed by tandem calpain D-III
domains. The knockout of Cpl in Dictyostelium generated similar phenotype of ablation of calpain 1 and calpain 2 in mammals.

Yeast two-hybrid screening of human liver library by using Gal4BD-calpain DIII X2 as bait revealed several positive clones, including flotillin-1. The positive cDNAs specifically interact with calpain-10 DIII X2, when tested with baits comprising the DIII domains from different calpains. It is likely that calpain-10 is correlated to type 2 diabetes through the lipid raft protein flotillin-1 which is involved in the insulin-stimulated glucose transport pathway.
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ABSTRACT

Calpains are intracellular, cysteine proteases found in vertebrates and some lower organisms. There is evidence that they are important mediators of cell adhesion and motility in animal cells. While there is evidence that the single nucleotide polymorphism in the non-coding region of the calpain 10 gene is associated with type 2 diabetes, little cellular and biochemical information is currently available on calpain 10. Because the cellular slime mold Dictyostelium discoideum is a genetically tractable model for cellular studies, in this organism we have investigated a calpain-like protein which may provide useful clues towards an understanding of the function of mammalian calpains. Contig 13130 (Sanger Institute Dictyostelium sequencing project) was identified as a 3-exon gene that encodes a calpain-like protein. Using a custom peptide antibody to assay for the presence of this putative protein, we identified Dictyostelium calpain-like protein (Cpl) and purified it to near homogeneity. Cpl is a 72,278 Da cytosolic protein. Weak caseinolytic activity inhibitable by cysteine protease inhibitors was co-purified with Cpl immunoreactivity, and purified Cpl appeared to undergo autoproteolysis when transferred to inhibitor-free buffer. The major cleavage after Pro189 generated a 51,291 Da Cpl fragment. Cpl domain structure resembles mammalian calpain 10 with a putative N-terminal catalytic domain followed by tandem calpain D-III domains. The knockout of Cpl in Dictyostelium showed similar phenotypes as ablation of calpain 1 and calpain 2 in mammalian cells. Yeast two-hybrid screening of a human fetal liver library by using Gal4BD-calpain DIIIX2 as bait revealed several positive clones, including flotillin-1. The proteins expressed by these clones specifically interacted with calpain 10 DIIIX2 when
tested with bait constructs comprising DIII domains from different calpains. Among the interacting proteins identified, flotillin-1 may be a promising subject for the relationship between calpain-10 and type 2 diabetes. Flotillin-1, and its binding proteins, CAP and cbl, are involved in insulin-induced glucose transport. It is possible that calpain-10 mediates the insulin-induced glucose transport pathway via its interaction with flotillin-1. Further confirmation of protein-protein interaction by co-immunoprecipitation and cellular function studies of the positive clones are under way.