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Characterization of Endogenous and Recombinant Human Calpain-10

Biao Dong and **Rihe Liu***
School of Pharmacy and Carolina Center for Genome Sciences, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

Abstract

Calpain-10 is a novel ubiquitous calpain family member that has been implicated as a susceptibility gene for type 2 diabetes. One of the major challenges is that the biochemical function of calpain-10 is not yet known. To address the problem, we purified human calpain-10 from different sources, including the endogenous and the recombinant calpain-10 from HeLa S3 and 293F cells, respectively. Both endogenous and recombinant calpain-10 were present as two major forms with different origins. Interestingly, radiolabeled calpain-10 was found to be efficiently cleaved at the N-terminal region by calpain-2, but not by other proteases. None of these calpain-10 proteins has putative proteolytic activity under *in vitro* conditions when examined using different peptide substrates, including more than 70 *in vitro* translated, radiolabeled oligopeptides. Our results raise the possibility that calpain-10 requires special intracellular localization or interacting partner(s) to acquire proteolytic activity, or it functions by interacting with other proteins rather than through its proteolytic activity.

Keywords

calpain-10; susceptibility gene for diabetes; calpain-10 isoforms; proteolytic activity; radiolabeled oligopeptides; random peptide library

INTRODUCTION

Calpain-10 is a new ubiquitous calpain family member that has been recently identified as the first candidate susceptibility gene for type 2 diabetes [1–5]. Since the first implication that genetic variations in the *CAPN10* gene are linked with increased risk of type 2 diabetes, many follow-up studies have been performed to verify the reported association in a number of ethnic populations. Intriguingly, varying levels of association between *CAPN10* and type 2 diabetes were reported. It appears that there is more than one *CAPN10* variant that could play a role in determining disease susceptibility, and the importance of different variants varies in different ethnic populations [5]. Due to these controversial results, functional studies on *CAPN10* in diabetes-related signaling pathways have drawn lots of attention. It was found that different variations in *CAPN10* were located in introns and could be associated with the change of expression level of CAPN10 mRNA [1,2]. In addition, these variations are also associated with diabetes related metabolic phenotypes, including free fatty acid level, insulin resistance, elevated triglyceride level, enhanced microvascular function, reduced β(3)-adrenoceptor

*To whom correspondence should be addressed Tel. (919) 843-3635; Fax: (919) 968-0081, E-Mail: rliu@email.unc.edu.

function in fat cells, blood glucose level, and polycystic ovary syndrome [6–11]. Recently, several new findings suggest that calpain-10 protein itself could be involved in insulin secretion and action. It was reported that calpain-10 might facilitate GLU4 vesicle translocation during insulin stimulated glucose uptake in adipocytes [12]. Furthermore, it was found that a short-term exposure to the cell-permeable calpain inhibitors increased the insulin secretory response to glucose in mouse pancreatic islets [13], whereas a 48-hour long-term exposure of mouse islets to calpain inhibitors reversibly suppressed glucose-induced insulin secretion [14].

One critical question that remains to be addressed is whether this protease domain-containing protein has the putative proteolytic activity? Two recent investigations provided important clues. First, a 54 kDa isoform of calpain-10 was found to directly interact with t-SNAREs, syntaxin 1, and SNAP-25 in pancreatic beta cells [15]. Significantly, SNAP-25 was found to undergo a Ca^{2+}-dependent partial proteolysis during stimulated secretion, raising the possibility that calpain-10 regulates insulin secretion through proteolytic cleavage of SNAP-25. More recently, two complex I proteins, namely NDUFV2 and ND6, were implicated as the potential downstream substrates of calpain-10 in mitochondrial [16]. In all these studies, the observed cleavage was indirectly attributed to calpain-10, presumably because the cleavage could be abolished by general calpain inhibitors. To biochemically characterize this protein, we purified calpain-10 from different sources, including the endogenous and the recombinant calpain-10 from HeLa S3 and 293F cells, respectively. Both endogenous and recombinant calpain-10 were present as two major forms, but with very different origins. We demonstrate that radiolabeled calpain-10 could be efficiently cleaved at the N-terminal region by calpain-2. However, none of these calpain-10 proteins has putative proteolytic activity when used for direct proteolytic analysis against various fluorogenic calpain substrates and more than 70 *in vitro* translated, radiolabeled oligopeptides. Our results raise the possibility that calpain-10 requires special intracellular localization or other interacting partner(s) to acquire proteolytic activity, or it functions by interacting with other proteins rather than through its proteolytic activity.

MATERIALS AND METHODS

Cell lines

Insect Sf9 cells from Novagen (Madison, WI) were grown at 27 °C as a semi-adherent culture in BacVector insect cell medium (Novagen). HeLa S3 cells from UNC tissue culture facility were maintained at 37 °C in a humidified incubator with 5% CO_2 and grown to the stationary phase in Ham's F12 medium supplemented with 10% fetal bovine serum. Human kidney 293F cells from Invitrogen (Carlsbad, CA) were cultured in a high glucose DMED medium with 1× NEAA, 2 mM Glutamin, 10% of fetal bovine serum, and 1× penicillin/streptomycin.

Cloning of the full-length human *CAPN10a* gene into different expression vectors

A cDNA clone (MGC-10770) containing the full-length human *CAPN10a* gene was obtained from ATCC (Manassas, VA) and used as the template for PCR amplification. To clone *CAPN10a* for expression in insect cells, the full-length coding sequence was PCR amplified using a high fidelity Platinum *Pfx* DNA polymerase (Invitrogen) and inserted into expression vector pIEx-1 Ek/LIC with N-terminal His×6- and S-tags according to the manufacturer's instructions (Novagen). To clone *CAPN10a* for expression in mammalian cells, the amplified coding sequence was inserted into expression vector pcDNA3.1D/V5-His TOPO according to the manufacturer's instructions (Invitrogen). The resulting *CAPN10a*-containing expression vectors with correct sequences were selected and confirmed by sequencing analysis.

Overexpression of calpain-10 in transfected Sf9 insect cells

Sf9 cells were transfected with pIEx-*CAPN10a* using Insect GeneJuice transfection reagent according to the manufacturer's instructions (Novagen). The cells were harvested at 48 hr after transfection and washed twice with ice-cold PBS, followed by cell lysis on ice in a buffer containing 50 mM Tris-HCl at pH 7.4, 150 mM NaCl, and 1% Triton X-100. After centrifugation at 14,000 rpm for 20 min at 4 °C, the supernatant was collected and the amount of total protein quantified. To detect the overexpressed calpain-10 present in the membranes, the insoluble fraction was dissolved in an SDS sample buffer (100 mM Tris-HCl at pH 6.8, 2% SDS, 0.01% bromophenol blue, 5 mM β-mercaptoethanol, and 10% glycerol) by heating at 95 °C for 5 min. The presence of recombinant human calpain-10 was detected by probing the Western blots using an anti-S tag antibody (Novagen) at 1:2000 dilution.

Overexpression and purification of calpain-10 in stably transfected 293F cells

293F cells were transfected with pcDNA3.1D/V5-His-*CAPN10a* using Lipofectamin 2000 (Invitrogen) according to the manufacturer's instructions. Selection of the stably transfected cell lines was initiated at 24 hr after transfection using a culture medium containing 750 μg/ml G418. The selection was accomplished during a period of 30 days and G418-resistant cell lines were maintained in the selective culture medium.

To purify overexpressed calpain-10a, stably transfected cells from 0.5 liter of culture were lysed in 12 mL of lysis buffer (50 mM NaH_2PO_4 at pH 8.0, 300 mM NaCl, 10 mM imidazole, and 1% Triton X-100) on ice for 30 min, followed by centrifugation at 14,000 rpm for 20 min. The supernatant was mixed with 0.6 mL of Ni-NTA agarose beads (Qiagen, Valencia, CA) at 4°C for 2 hr and the mixture loaded into an empty Bio-Rad column for separation. After washing the column 3 times with 15 mL of wash buffer (50 mM NaH_2PO_4 at pH 8.0, 300 mM NaCl, 20 mM imidazole, and 0.05% Tween 20), the bound protein was eluted using 3.0 mL of elution buffer (50 mM NaH_2PO_4 at pH 8.0, 300 mM NaCl, 250 mM imidazole, and 0.05% Tween 20). The eluate was dialyzed in a buffer containing 10 mM Tris-HCl at pH 7.4, 50 mM NaCl, 1 mM EDTA, and 0.05% Tween 20. All purification steps were performed at 4 °C. Calpain-10 thus purified was confirmed by Western blot analysis using anti-V5 antibody (Invitrogen) at 1:3000 dilution.

To quantify the amount of protein by silver staining, the SDS-PAGE gel was first fixed in a fixation solution (50% ethanol and 5% acetic acid in water). After wash and incubation in a sensitizing solution (0.02% sodium thiosulphate), the gel was stained using a solution of 0.1% silver nitrate at 4 °C for 30 min, prior to the addition of a developing solution (0.04% formalin and 2% sodium carbonate). Upon the appearance of the desired protein bands, the gel was incubated with 5% acetic acid for 5 min. Different amounts of highly purified BSA were also loaded on the gel as the standard for protein quantification.

Purification of endogenous calpain-10 from HeLa S3 cells using immunoprecipitation

Suspension HeLa S3 cells were cultured in Vented-cap shakerflasks on an orbital shaker at 120–150 rpm. After washing, cells were homogenized on ice for 15 min in a TBS buffer (50 mM Tris-HCl at pH 7.4, 150 mM NaCl). The supernatant was collected by centrifuging at 4 ° C for 20 min. The amount of total protein was quantified using Coomassie Plus protein assay (Bio-Rad, Hercules, CA). To immunoprecipitate the endogenous calpain-10, each cell lysate with 1 mg of total protein was precleared with 20 μl of Protein A/G Plus-agarose (Santa Cruz Biotech, Santa Cruz, CA) at 4 °C for 30 min, followed by incubation with 5 μg of anti-calpain-10 antibody that recognizes the N-terminal domain I (Sigma, St. Louis, MO). 50 μl of protein A/G Plus-agarose was then added and the reaction mixture was continuously rotated at 4 °C for 2 hr. After washing, the immunoprecipitated calpain-10 was eluted with 100 μl of 200 μM peptide that was used to raise the antibody (Triple Point Biologics, Forest Grove, OR).

Various anti-calpain-10 antibodies were used to detect the presence of calpain-10 isoforms, including anti-DI (Sigma, St. Louis, MO), anti-DC (Sigma), and anti-DT (Santa Cruz Biotechnology, Santa Cruz, CA) that recognize the N-terminal domain I, the catalytic domain, and the C-terminal T domain of calpain-10, respectively.

Cloning and expression of radiolabeled oligopeptides or full-length proteins as substrates for proteolytic analysis

Colonies from a DNA library coding for combinatorial synthetic peptides were randomly picked up and their coding regions amplified by PCR [17]. Full length human SNAP-25, NDUFV2, and ND6 genes were PCR amplified from a human bone marrow cDNA library (Invitrogen). The primers for SNAP-25 were 5′-CAATTACTATTTACAATTACAATGGCCGAAGACGCA-3′ and 5′-TTAATGGTGATG GTGATGATGACCACTTCCCAGCAT-3′; the primers for NDUFV2 were 5′-CAATTACTATTTACAATTACAATGGGATTCTTCTCCGCGGCGC-3′ and 5′-TTAATGGTGATGGTGATGATGAAGGCCTGCTTGTACACCAAATC-3′; and the primers for ND6 were 5′-CAATTACTATTTACAATTACAATGGGAATGTATGCTTTGTTTCTGTTG AGTG-3′ and 5′-TTAATGGTGATGGTGATGATGATTCCCCCGAGCAATCTCAATTAC-3′. Each PCR product was further amplified using a pair of second primers to introduce T7 promoter and TMV 5′ UTR for *in vitro* transcription and translation, respectively.

The final PCR products were purified and used as templates to generate [^{35}S]-methionine labeled full-length proteins by coupled *in vitro* transcription and translation (TNT). A typical TNT reaction mixture contains 1× translation buffer without methionine, 75 mM KOAc, 5.5 mM $Mg(OAc)_2$, 2 mM each of rNTPs, 1 mM spermidine, 0.03 U/μl of T7 RNA polymerase, 5 μCi [^{35}S]-methionine, 180 ng of purified PCR product, and 10 μl of rabbit reticulocyte lysate (Novagen). The reaction was performed by incubating at 30 °C for 1.5 hr and the mixture stored at −20 °C for further use.

Proteolytic analysis of calpain activity using fluorogenic short peptide substrates or radiolabeled oligopeptide substrates

Commercially available fluorogenic calpain substrates Suc-LY-AMC (Calbiochem, San Diego, CA) and Suc-LLVY-AMC (Biomol, Plymouth Meeting, PA) were used in the calpain activity assay. The reaction was carried out at 37 °C for 1 hr in a reaction buffer containing 50 mM Tris-HCl at pH 7.4, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM β-mercaptoethanol, 5 mM $CaCl_2$, and 100 μM fluorogenic substrate. The reaction was initiated by adding approximately 250 ng of a purified calpain and the signal was measured using an LS50B luminescence spectrometer (PerkinElmer, Waltham, MA) with excitation and emission at 380 nm and 460 nm, respectively. A reaction mixture with human calpain-1 or rat calpain-2 (Calbiochem, San Diego, CA) was used as the positive control, whereas a reaction mixture without any protease was used as the negative control.

Protease activity assay was also carried out using endogenous or recombinant human calpain-10 purified from cultured cells in the presence of an exogenous lipid vesicle. Different lipid vesicles were used, each containing 1% (w/w) phosphatidylcholine with 1% (w/w) N-(biotinoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine. We also used liposomes contain either 5% (w/w) PI(3)P, PI(4)P, $PI(3,4)P_2$, $PI(4,5)P_2$, or $PI(3,4,5)P_3$, respectively. Liposomes were prepared using the methods reported in the literature [18,19]. Briefly, appropriate amounts of each lipid in chloroform were mixed and dried under nitrogen. The lipid mixture was resuspended in TBS buffer by vortexing and the liposomes were generated by sonication.

To get a large number of oligopeptides with different sequences by TNT in rabbit reticulocyte lysate, individual clones from a high quality DNA library were used as templates [17]. Each sequence in the DNA library codes for 53 amino acids, with 20 randomized residues in the middle cassette flanked by affinity tags at both the N- and the C-termini. The DNA library was cloned and more than 100 colonies were picked up for sequencing. 72 unique sequences were PCR amplified and used as templates to synthesize the corresponding radiolabeled oligopeptides. To perform *in vitro* proteolytic analysis using radiolabeled oligopeptides as the substrates, an aliquot of each TNT reaction mixture was incubated at 30 °C with ~100 ng of purified calpain-10 for 6 hr or with 50 ng of rat calpain-2 for 2 hr as control. After digestion, an aliquot of each reaction mixture was loaded onto an SDS-PAGE gel for separation and the signal was detected by autoradiography.

In vitro Ca^{2+}-dependent autolytic assay

To initiate autolysis, 300 ng of Ni-NTA-purified, recombinant calpain-10 from 293F cells or immunoaffinity-purified endogenous calpain-10 from HeLa S3 cells was incubated in a buffer containing 50 mM Tris-HCl at pH 7.4, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 5 mM β-mercaptoethanol. The reaction was initiated by adding $CaCl_2$ to 5 mM (total Ca^{2+} concentration). The reaction was performed at 30 °C for 2 hr and terminated by adding SDS-PAGE sample buffer. Western blot analysis was carried out using anti-V5 or anti-calpain-10 antibodies.

In vitro proteolysis of calpain-10 by different proteases

The full-length calpain-10 and its N-terminal truncated fragment (residues 291–672) were generated by TNT in the presence of [^{35}S]-methionine. To perform proteolytic analysis of radiolabeled calpain-10 proteins, an aliquot of the TNT reaction mixture was incubated at 30 °C for 2 hr with an appropriate amount of a protease of interest, including caspases (Biovision, Mountain View, CA), calpain-1 (EMD Chemicals, San Diego, CA), calpain-2 (EMD Chemicals), purified calpain-10 or no protease as control. ALLN (EMD Chemicals) was used as the general calpain inhibitor. The reaction was stopped by the addition of SDS-PAGE sample buffer. An aliquot of each reaction mixture was loaded onto an SDS-PAGE gel for separation, and the signal was detected by autoradiography.

RESULTS

Endogenous human calpain-10 in HeLa S3 cells were present as two major isoforms with intact N-terminus but different C-terminal T domains

Human *CAPN10a* gene consists of 15 exons and undergoes alternative splicing to generate a protein family of 8 distinct isoforms [1]. The isoforms that contain a complete protease domain are calpain-10a, -10b, -10c, and -10d. RT-PCR analysis indicated that the mRNA of calpain-10a was the most abundant isoform in various tissues and that of calpain-10c was easy to detect, but mRNAs of calpain-10b and -10d were present at much less abundance [1]. Calpain-10a and -10c have 672 and 517 amino acids, respectively, with identical sequences upstream of the domain III. It is not yet known whether the removal of the N-terminal region of calpain-10 is necessary for its activation, and where the cleavage site is if the N-terminus should indeed be processed. Therefore, we first examined the endogenous calpain-10 protein in HeLa S3 and 293 cells. In general, the expression patterns of calpain-10 in HeLa S3 and 293 cells were similar but not identical. Two major protein bands were detected on the gel when the Western blots were probed using an antibody that targets the N-terminal domain I (anti-DI), with molecular weight at approximately 74 kDa and 60 kDa, respectively (Figure 1A, right panel). This result indicates that the N-terminal domain is present in both proteins. By comparing the intensity of these two bands, it appears that the 60 kDa protein was the dominant one. The top band is most likely from the full-length calpain-10a based on its molecular weight.

To examine the nature of the 60 kDa protein, the Western blots were further probed with antibodies that recognize the middle catalytic domain (anti-DC) and the C-terminal T domain (anti-DT), respectively. Interestingly, it was found that this protein could be detected by anti-DC (data not shown), but not by anti-DT that recognizes a calpain-10a specific region (residues 530–550) close to the C-terminus (Figure 1A, left panel). Therefore, the 60 kDa protein should be another isoform, most likely calpain-10c, instead of the proteolytic product of calpain-10a at the N-terminus. Indeed, its size on SDS-PAGE gel correlates well with the molecular weight of calpain-10c that has 517 amino acids.

Another protein band with MW at 45 kDa was also detected in both cell lysates (Figure 1A). Since the antigen sequence (residues 530–550) recognized by anti-DT is only present on calpain-10a but not on any other calpain-10 isoforms, it appears that this 45 kDa protein is derived from calpain-10a. Several faint protein bands were observed in 293 cells but not in HeLa S3 cells when the blots were probed with anti-DI, suggesting the presence of other calpain-10 isoforms in 293 cells.

To obtain enough endogenous calpain-10 for proteolytic analysis, suspension HeLa S3 cells were grown in a large scale. The endogenous calpain-10 was purified by immunoprecipitation using the anti-DI antibody, followed by a competitive elution with the peptide that was used to raise the antibody. Endogenous calpain-10 with a molecular weight of 74 kDa was efficiently isolated with high purity (Figure 1B, lanes 4), which corresponds to the full-length calpain-10a. It is worth mentioning that although the 60 kDa protein could be detected on SDS-PAGE gel with anti-DI, it could not be captured by the same antibody when immunoprecipitation was performed under native conditions. One possible explanation is that the N-terminus of this 60 kDa calpain-10c is less accessible compared to that of the 74 kDa calpain-10a.

Most of the human calpain-10 protein overexpressed in insect cells was present in the membrane-bound fraction

It is well known that active calpains are very difficult to overexpress in bacteria [20–26]. However, calpains expressed in the baculovirus-insect cell system were at least partially soluble and active [27–29]. It has been reported that rat calpain-10 could be overexpressed in insect cells, although detailed biochemical characterization was not reported [30]. Therefore, we first expressed full-length calpain-10a in insect Sf9 cells using the commercially available pIEx expression vector that features an hr5 enhancer and an IE1 promoter to direct efficient expression in insect cells without creating a recombinant baculovirus [31]. Indeed, the recombinant calpain-10 was expressed as a protein with a single band at 79 kDa, a molecular weight close to that of the full-length calpain-10a with the engineered affinity tags (data not shown). However, the expression level of the soluble calpain-10a in Sf9 cells was quite low, while most of the recombinant protein was present in the membrane-bound fraction. Since it is very difficult to remove other endogenous membrane-bound proteases, we did not use calpain-10 thus generated for proteolytic studies.

Human calpain-10 protein overexpressed in 293F cells was present as two forms that were intact at both N- and C-termini

We then expressed human calpain-10 in mammalian cells. Since the N-terminus of calpain-10 might be sensitive for autolytic processing, we introduced the V5/His tags at its C-terminus while keeping the N-terminus unmodified, except the addition of a glycine residue after the first methionine to facilitate overexpression. This construct is different from prior report in which the *CAPN10a* gene was inserted into a pcDNA3.1/HisA vector with His/Xpress tags at the N-terminus [15]. The construct was used to transfect human 293F suspension cells. A total of 120 stably transfected cell lines were selected, and the one that showed the highest expression level (293F-E3) was used for subsequent calpain-10 expression (Figure 2A, lane 9). To rule

out the possibility that mutations might be introduced in the inserted calpain-10a gene during selection for protein expression in the stably transfected 293F cells, the integrated calpain-10a gene in E3 cells was PCR amplified and sequenced. It appears that the inserted gene is intact without any mutations.

Two protein bands were also detected on SDS-PAGE gel from almost all the clones we have characterized, with molecular weight at approximately 79 kDa and 62 kDa, respectively, when the Western blots were probed with anti-V5 that targets the V5 tag engineered at the C-terminus (Figure 2A). The expression level of the 62 kDa protein was higher than that of the 79 kDa protein among all the clones we have analyzed. Interestingly, both the expression level and the ratio of the top and bottom bands were highly dependent on the status of the cells. Figure 2B shows that the expression level of the full-length calpain-10 in 293F cells was very low, and the major form was the 62 kDa protein when the cells were in their fast growing status. However, the expression level of both forms were similar when the cells were growing slowly. This result implies that full-length calpain-10a might be toxic to cell growth and/or be degraded efficiently. To explore whether the disappearance of the top lane could be due to proteasome-mediated degradation, overexpression was carried out in the presence of a 26S proteasome inhibitor MG-132. No difference was detected when such an inhibitor was present (data not shown), suggesting that the degradation of the 79 kDa calpain-10 is less likely through the UPS pathway.

The size of the 79 kDa protein band fits well with that of the full-length calpain-10a with C-terminal V5/His tags. Since we used the ORF of full-length *CAPN10a* gene lack of introns for stable transfection, the 62 kDa recombinant protein should be derived from the calpain-10a instead of from other isoforms. To examine the origin of this 62 kDa protein, the recombinant proteins were probed with antibodies that recognize different calpain-10 domains. First, both proteins were recognized by anti-V5 and anti-His×6 that target the affinity tags engineered at the C-terminus. Second, both the 79 kDa and the 62 kDa bands were detected when the blots were probed with either the anti-DC that recognizes the middle of the catalytic domain or with the anti-DT that recognizes calpain-10a specific T-domain, indicating the presence of both the catalytic and the T domains (data not shown). Based on these results, the 62 kDa recombinant protein observed here is different from the 60 kDa calpain-10c isoform that could not be recognized by calpain-10a specific anti-DT (Figure 1A, left panel).

Interestingly, both the top and the bottom bands were also clearly recognized when the blots were probed using an antibody that recognizes domain I (Figure 2C), indicating that the 62 kDa calpain-10 has an intact N-terminus and therefore should not be the N-terminal processed product. Indeed, the molecular weight difference of these two calpain-10 forms is approximately 15–19 kDa, which cannot be explained by the removal of the 41-residue domain I. Interestingly, Marshall and coworkers also observed that the recombinant calpain-10 in cytosol was present as multiple forms with similar molecular weights, which were detected by using anti-calpain-10 antibody [15]. Since different antibodies were used, it is difficult to compare the results from different studies. Based on our experiments, it appears that the 62 kDa recombinant protein is a calpain-10 that has both intact domain I and domain T, but not the domain I-processed product of calpain-10a. We further investigated the expression of such recombinant calpain-10 using transient instead of stable transfection. Interestingly, the same two forms of calpain-10 were also observed, indicating they were not caused by stable transfection. Our results suggest that these two calpain-10 forms differ at internal site(s). One possibility is that there is an unidentified, yet translatable intron in the cDNA of calpain-10a, which might be processed when overexpression of calpain-10 was attempted. However, we were not yet able to prove this mechanism. Ideally, the nature of different forms of calpain-10 discussed here should be confirmed by Mass spectrometry. However, our efforts in using Mass spectrometry to determine the molecular weighs of these calpain-10 proteins did not result in

high quality data, presumably because the calpain-10 proteins thus generated were not pure enough for this purpose.

Proteolytic assay using *in vitro* translated, radiolabeled oligopeptide substrates

Since it is not yet known which recombinant calpain-10 has the putative activity, we purified both forms using Ni-NTA resin that can capture the His×6 tag engineered at the C-terminus. To address the possible loss of the protease activity of calpain-10, the purification was carefully performed under conditions that should minimize protein unfolding and denaturing. The His×6 tag-based purification was efficient, with more than 60% of the recombinant calpain-10 recovered from the eluate, as indicated by Western blotting using anti-V5 antibody (Figure 2D). With the availability of both endogenous and recombinant calpain-10 proteins, we systematically examined their proteolytic activity under various conditions. First, purified calpain-10 proteins were incubated under various conditions that facilitate autolysis, in the presence or absence of Ca^{2+}. Since both endogenous and recombinant calpain-10 were present as two forms, the autolysis was carefully monitored by the disappearance of the top band and the increase of the bottom band or the appearance of other smaller protein bands on SDS-PAGE gel. However, we were not able to detect any obvious changes (Figure 3A), indicating that the N-terminal autolysis did not occur under the conditions we have examined. Purified calpain-10 proteins were also used to cleave two widely used commercially available fluorogenic calpain substrates (Suc-LY-AMC and Suc-LLVY-AMC). No cleavage was observed in any of the fluorogenic assays we have carried out (data not shown).

Calpain family members are often dependent upon membrane association to facilitate proteolytic activity. Since it is difficult to purify membrane-bound calpain-10, we addressed the problem by conducting *in vitro* proteolysis in conjuction with a variety of exogenous lipid/liposome supplements. To this end, different lipid vesicles were used to provide an artificial membrane that might serve as a catalyst for calpain-10 activation. Nevertheless, no calpain-10 activation was observed in the presence of the lipid/liposome we examined. We also examined the activity of *in vitro* translated calpain-10 generated by TNT. No protease activity could be detected when fluorogenic calpain substrates were used, consistent with the result when a potential calpian-10 substrate SNAP 25 was cotranslated in the same lysate (data not shown).

It is well known that calpain-1 and calpain-2 have optimal activities when present as a heterodimeric form with a 30 kDa small subunit, although the recombinant calpain-3 and the large subunits of calpain-1 and -2 expressed in baculovirus system retained substantial activity when the small subunit was absent [21,23,24,28]. Structurally, calpain-10 is less likely to interact with the small subunits of calpains, since its domain IV is a T domain instead of a calmodulin-like domain that is critical for heterodimerization as observed in other calpain members [1,4]. To address the possible involvement of other interacting partner(s), an ideal approach will be to add to the reaction mixture a calpain-depleted human cell lysate to complement the required binding partner(s). However, most human cell lysates contain numerous proteases including various calpain members that are impossible to completely deplete. Therefore, we chose to use rabbit reticulocyte lysate whose endogenous protease activity is below the detection limit. Rabbit reticulocyte lysate is from reticulocyte cells that contain most proteins in mammalian cells and therefore an excellent source for potential interacting partner(s). Another barrier to examining calpain-10 activity is lack of a known substrate. To address the problem, we synthesized a large number of oligopeptides with different sequences through coupled *in vitro* transcription and translation (TNT). The use of rabbit reticulocyte lysate has an additional advantage of convenient synthesis of the radiolabeled oligopeptide substrates in the same lysate. Furthermore, the cleavage of each oligopeptide substrate could be readily detected through autoradiography, which has a sensitivity hundred times higher than that of the conventional fluorogenic approach.

Altogether, this system is particularly suitable for examining a protease whose proteolytic activity is either unknown or very low.

We first generated by TNT 72 oligopeptides in rabbit reticulocyte lysate. Each oligopeptide sequence is unique. The amino acid sequences of the random region of these 72 oligopeptides are listed in Supplementary Table 1. The combination of these 72 oligopeptides should include a large number of potential cleavage sites, which are impossible to cover by a limited number of commercially available fluorogenic calpain substrates. In addition, the oligopeptides we used here are much longer than the conventional fluorogenic substrates, and therefore more likely to have a secondary structure that could facilitate recognition by calpains [32,33].

To perform *in vitro* proteolytic analysis using radiolabeled oligopeptide substrates, the product of each TNT reaction was treated either with a purified (endogenous or recombinant) calpain-10 or with a control eluate. We first validated this assay by using calpain-1 and calpain-2 as positive controls. It was found that most of the oligopeptide substrates were cleaved efficiently, as shown in Figure 3B as an example. We then used purified calpain-10 to digest each of the 72 oligopeptides. Since it is not yet known which recombinant calpain-10 form has the desired protease activity, we used a mixture of both the longer and the shorter forms of calpain-10 for proteolysis. Figure 3C illustrates that the quantity of most oligopeptides was not changed after incubation with purified calpain-10, compared with the corresponding negative control. We also performed the proteolytic reaction in the presence of an artificial membrane, including microsomal membrane. No proteolytic activity was detected when the membrane was present.

The radiolabeling nature of the oligopeptide substrates makes the assay very sensitive, and should allow the detection of proteolytic activity even at very low levels. Indeed, all the radiolabeled substrates were very efficiently cleaved by calpain-1 or -2 through a short digestion, but not by calpain-10 even after a long incubation. Since the oligopeptide substrates used in the proteolytic analysis contained a large number of possible combinations, the lack of putative proteolytic activity suggests that either calpain-10 require special intracellular localization or interacting partner(s) to acquire proteolytic activity, or its low proteolytic activity under *in vitro* conditions cannot be detected unless a much more sensitive assay is adopted.

Radiolabeled calpain-10 was efficiently cleaved at the N-terminal region by calpain-2

It is likely that the unnatural peptide substrates used here are not suitable for examining its proteolytic activity, and protein substrates closer to known calpain motifs or calpian-10 itself should be used. To this end, we synthesized calpain-10 and its N-terminal truncated fragment (residues 291–672) by using TNT. These radiolabeled calpain-10 proteins were incubated with calpain-1, calpain-2, purified calpain-10, and other common proteases including various caspases. Figure 4A shows that full-length calpain-10 was efficiently cleaved by calpain-2, but not by calpain-1, purified calpain-10, or caspases (data not shown). It appears that the cleavage occurs at the N-terminus, since the N-terminal truncated fragment was not digested under the same conditions.

Calpain-10 substrates implicated in the literature were cleaved by calpain-1 and calpain-2 but not by purified calpain-10

Recently, several proteins were implicated as potential calpain-10 substrates, including SNAP-25, NDUFV2, and ND6 [15,16]. These proteins were found to be cleaved *in vivo* in a calpain inhibitor-dependent manner. However, it is difficult to determine which member in the calpain family was responsible for the observed cleavage. To investigate whether these three proteins were specifically cleaved by calpain-10, we generated by TNT their full-length in the

presence of [^{35}S]-methionine and used them as the substrates with purified calpain-10 or rat calpain-2 as control. As illustrated in Figure 4B, all three proteins were efficiently cleaved by rat calpain-2 in a Ca^{2+}-dependent manner. Such cleavage was completely abolished by chelating Ca^{2+} with EGTA or by adding a general calpain inhibitor ALLN. These proteins were also efficiently cleaved by calpain-1, while none of them was digested by purified calpain-10 (data not shown). To examine whether calpain-10 thus processed was active, we incubated calpain-2 with purified calpain-10 and measured the proteolytic activity of the reaction mixture by comparing the overall activity in the presence or absence of calpain-10. No difference was detected, suggesting calpain-10 thus processed is not active.

DISCUSSION

In this work, we report that both endogenous and recombinant calpain-10 were present as two major forms but with very different origins. We found that several calpain-10 substrates implicated in the literature were cleaved by calpain-1 and calpain-2 but not by purified calpain-10. It is worth mentioning that this result was obtained by using purified calpain-10 under *in vitro* conditions. Since calpain-10 could be active only under *in vivo* conditions or at specific intracellular locations, the implicated cleavage is still likely to occur under physiological conditions [15,16]. Therefore, it is of great importance to investigate the biological functions of calpain-10 under *in vivo* conditions.

Our results indicate that the proteolytic activity of both endogenous and recombinant calpain-10 could not be confirmed under *in vitro* conditions. There are numerous possible explanations for the failure to detect the putative proteolytic activity. It is likely that the activity was lost during purification, although all the expression and purification procedures were carefully carried out under conditions that should maintain the putative protease activity. Due to the lack of a specific enzymatic assay for calpain-10, we were not able to monitor the enzymatic activity of calpain-10 at each purification step.

It is also likely that the biologically active isoform of calpain-10 was not purified. It has been implicated that the *in vivo* activity of calpain may be regulated through membrane association, although several calpains expressed in baculovirus system have desired proteolytic activity in the absence of membrane. Under normal physiological conditions, calpain-1 and -2 are not membrane-bound, but an increasing fraction was found to become membrane-associated with the increase of the intracellular Ca^{2+} concentration [34]. When overexpressed in insect Sf9 cells, the amount of human calpain-10 in the insoluble fraction was much higher than that in the soluble fraction. This result was consistent with previous report that the highest concentration of calpain-10 was in the insoluble fraction of tissues [30]. It is likely that calpain-10 requires membrane association to gain proteolytic activity. However, it is very challenging to purify a membrane fraction that contains only calpain-10, presumably because the membrane fraction usually contains other cellular proteases, making it difficult to attribute the proteolytic activity to calpain-10. The membrane-bounded 54 kDa calpain-10 isoform has been implicated to be the functional protease for the cleavage of SNAP-25 [15]. However, we were not able to purify an isoform with that molecular weight using the antibodies we have tried. Furthermore, the use of exogenous lipid/liposomes in the proteolytic reaction could not activate calpain-10. Another critical step that could affect calpain activity is the autolysis of the N-terminal domain I, which varies in different calpain members. It has been shown that some N-terminal mutations of the large subunit of calpain 1 prevented only autolysis but not the cleavage of substrates, and non-autolyzed calpain was still able to cleave substrates [22, 34]. It is not yet known whether autolysis is indispensable for ite proteolytic activity of calpain-10. Future work should be directed to examine the proteolytic activity of membrane-bound calpain-10 with different N-terminal truncations.

It is worth mentioning that the results from this study are only limited to the human calpain-10 expressed under the conditions we have examined. It is likely that calpain-10 in different intracellular locations such as that in mitochondria could have different properties [16,35]. Although we were not able to confirm the putative protease activity of calpain-10, our results imply that the activation of calpain-10 might occur at specific intracellular locations or under unique physiological conditions when its regulatory proteins are available. It is also possible that calpain-10 is a protein whose major biochemical functions are achieved by interacting with other proteins rather than through its proteolytic activity.

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ABBREVIATIONS

ALLN
calpain inhibitor Ac-Leu-Leu-Nle-H

CAPN1
calpain-1 gene

CAPN10
calpain-10 gene

DMED
Dulbecco's Modified Eagle's Medium

GLU4
glucose transporter member 4

NDUFV2
NADH dehydrogenase (ubiquinone) flavoprotein 2

ND6
NADH dehydrogenase subunit 6

NEAA
non-essential amino acids

Ni-NTA
Ni^{2+}-nitrilotriacetic acid

PBS
phosphate buffered saline

SDS
sodium dodecyl sulfate

SNARE
soluble NSF attachment receptor

SNAP-25
synaptosome-associated protein of 25 kDa

TBS

Tris buffered saline

TMV

tobacco mosaic virus

TNT

coupled *in vitro* transcription and translation

UPS

ubiquitin-proteasome system

UTR

un-translated region

References

1. Horikawa Y, Oda N, Cox NJ, Li X, Orho-Melander M, Hara M, Hinokio Y, Lindner TH, Mashima H, Schwarz PE, del Bosque-Plata L, Oda Y, Yoshiuchi I, Colilla S, Polonsky KS, Wei S, Concannon P, Iwasaki N, Schulze J, Baier LJ, Bogardus C, Groop L, Boerwinkle E, Hanis CL, Bell GI. Genetic variation in the gene encoding calpain-10 is associated with type 2 diabetes mellitus. Nat Genet 2000;26:163–175. [PubMed: 11017071]
2. Baier LJ, Permana PA, Yang X, Pratley RE, Hanson RL, Shen GQ, Mott D, Knowler WC, Cox NJ, Horikawa Y, Oda N, Bell GI, Bogardus C. A calpain-10 gene polymorphism is associated with reduced muscle mRNA levels and insulin resistance. J Clin Invest 2000;106:R69–73. [PubMed: 11018080]
3. Cox NJ. Calpain 10 and genetics of type 2 diabetes. Curr Diab Rep 2002;2:186–190. [PubMed: 12643138]
4. Huang Y, Wang KK. The calpain family and human disease. Trends Mol Med 2001;7:355–362. [PubMed: 11516996]
5. Turner MD, Cassell PG, Hitman GA. Calpain-10: from genome search to function. Diabetes Metab Res Rev 2005;21:505–514. [PubMed: 16028216]
6. Orho-Melander M, Klannemark M, Svensson MK, Ridderstrale M, Lindgren CM, Groop L. Variants in the calpain-10 gene predispose to insulin resistance and elevated free fatty acid levels. Diabetes 2002;51:2658–2664. [PubMed: 12145185]
7. Carlsson E, Fredriksson J, Groop L, Ridderstrale M. Variation in the calpain-10 gene is associated with elevated triglyceride levels and reduced adipose tissue messenger ribonucleic acid expression in obese Swedish subjects. J Clin Endocrinol Metab 2004;89:3601–3605. [PubMed: 15240652]
8. Shore AC, Evans JC, Frayling TM, Clark PM, Lee BC, Horikawa Y, Hattersley AT, Tooke JE. Association of calpain-10 gene with microvascular function. Diabetologia 2002;45:899–904. [PubMed: 12107735]Epub 2002 May 2015
9. Hoffstedt J, Naslund E, Arner P. Calpain-10 gene polymorphism is associated with reduced beta(3)-adrenoceptor function in human fat cells. J Clin Endocrinol Metab 2002;87:3362–3367. [PubMed: 12107250]
10. Lynn S, Evans JC, White C, Frayling TM, Hattersley AT, Turnbull DM, Horikawa Y, Cox NJ, Bell GI, Walker M. Variation in the calpain-10 gene affects blood glucose levels in the British population. Diabetes 2002;51:247–250. [PubMed: 11756349]
11. Gonzalez A, Abril E, Roca A, Aragon MJ, Figueroa MJ, Velarde P, Royo JL, Real LM, Ruiz A. Comment: CAPN10 alleles are associated with polycystic ovary syndrome. J Clin Endocrinol Metab Endocrinol 2002;87:3971–3976.
12. Paul DS, Harmon AW, Winston CP, Patel YM. Calpain facilitates GLUT4 vesicle translocation during insulin-stimulated glucose uptake in adipocytes. Biochem J 2003;376:625–632. [PubMed: 12974673]
13. Sreenan SK, Zhou YP, Otani K, Hansen PA, Currie KP, Pan CY, Lee JP, Ostrega DM, Pugh W, Horikawa Y, Cox NJ, Hanis CL, Burant CF, Fox AP, Bell GI, Polonsky KS. Calpains play a role in insulin secretion and action. Diabetes 2001;50:2013–2020. [PubMed: 11522666]

14. Zhou YP, Sreenan S, Pan CY, Currie KP, Bindokas VP, Horikawa Y, Lee JP, Ostrega D, Ahmed N, Baldwin AC, Cox NJ, Fox AP, Miller RJ, Bell GI, Polonsky KS. A 48-hour exposure of pancreatic islets to calpain inhibitors impairs mitochondrial fuel metabolism and the exocytosis of insulin. Metabolism 2003;52:528–534. [PubMed: 12759879]
15. Marshall C, Hitman GA, Partridge CJ, Clark A, Ma H, Shearer TR, Turner MD. Evidence that an isoform of calpain-10 is a regulator of exocytosis in pancreatic beta-cells. Mol Endocrinol 2005;19:213–224. [PubMed: 15471947]Epub 2004 Oct 2007
16. Arrington D, Van Vleet T, Schnellmann R. Calpain 10: A Mitochondrial Calpain and its Role in Calcium-induced Mitochondrial Dysfunction. Am J Physiol Cell Physiol 2006;21:21.
17. Cho G, Keefe AD, Liu R, Wilson DS, Szostak JW. Constructing high complexity synthetic libraries of long ORFs using in vitro selection. J Mol Biol 2000;297:309–319. [PubMed: 10715203]
18. Zhu H, Bilgin M, Bangham R, Hall D, Casamayor A, Bertone P, Lan N, Jansen R, Bidlingmaier S, Houfek T, Mitchell T, Miller P, Dean RA, Gerstein M, Snyder M. Global analysis of protein activities using proteome chips. Science 2001;293:2101–2105. [PubMed: 11474067]
19. Casamayor A, Torrance PD, Kobayashi T, Thorner J, Alessi DR. Functional counterparts of mammalian protein kinases PDK1 and SGK in budding yeast. Curr Biol 1999;9:186–197. [PubMed: 10074427]
20. DeLuca CI, Davies PL, Samis JA, Elce JS. Molecular cloning and bacterial expression of cDNA for rat calpain II 80 kDa subunit. Biochim Biophys Acta 1993;1216:81–93. [PubMed: 8218419]
21. Graham-Siegenthaler K, Gauthier S, Davies PL, Elce JS. Active recombinant rat calpain II. Bacterially produced large and small subunits associate both in vivo and in vitro. J Biol Chem 1994;269:30457–30460. [PubMed: 7982961]
22. Elce JS, Hegadorn C, Gauthier S, Vince JW, Davies PL. Recombinant calpain II: improved expression systems and production of a C105A active-site mutant for crystallography. Protein Eng 1995;8:843–848. [PubMed: 8637855]
23. Vilei EM, Calderara S, Anagli J, Berardi S, Hitomi K, Maki M, Carafoli E. Functional properties of recombinant calpain I and of mutants lacking domains III and IV of the catalytic subunit. J Biol Chem 1997;272:25802–25808. [PubMed: 9325309]
24. Meyer SL, Bozyczko-Coyne D, Mallya SK, Spais CM, Bihovsky R, Kaywooya JK, Lang DM, Scott RW, Siman R. Biologically active monomeric and heterodimeric recombinant human calpain I produced using the baculovirus expression system. Biochem J 1996;314:511–519. [PubMed: 8670065]
25. Masumoto H, Yoshizawa T, Sorimachi H, Nishino T, Ishiura S, Suzuki K. Overexpression, purification, and characterization of human m-calpain and its active site mutant, m-C105S-calpain, using a baculovirus expression system. J Biochem (Tokyo) 1998;124:957–961. [PubMed: 9792919]
26. Hitomi K, Uchiyama Y, Ohkubo I, Kunimatsu M, Sasaki M, Maki M. Purification and characterization of the active-site-mutated recombinant human mu-calpain expressed in baculovirus-infected insect cells. Biochem Biophys Res Commun 1998;246:681–685. [PubMed: 9618272]
27. Lee HJ, Tomioka S, Kinbara K, Masumoto H, Jeong SY, Sorimachi H, Ishiura S, Suzuki K. Characterization of a human digestive tract-specific calpain, nCL-4, expressed in the baculovirus system. Arch Biochem Biophys 1999;362:22–31. [PubMed: 9917325]
28. Branca D, Gugliucci A, Bano D, Brini M, Carafoli E. Expression, partial purification and functional properties of themuscle-specific calpain isoform p94. Eur J Biochem 1999;265:839–846. [PubMed: 10504417]
29. Fukiage C, Nakajima E, Ma H, Azuma M, Shearer TR. Characterization and regulation of lens-specific calpain Lp82. J Biol Chem 2002;277:20678–20685. [PubMed: 11904300]Epub 22002 Mar 20619
30. Ma H, Fukiage C, Kim YH, Duncan MK, Reed NA, Shih M, Azuma M, Shearer TR. Characterization and expression of calpain 10. A novel ubiquitous calpain with nuclear localization. J Biol Chem 2001;276:28525–28531. [PubMed: 11375982]Epub 22001 May 28525
31. Jarvis DL, Weinkauf C, Guarino LA. Immediate-early baculovirus vectors for foreign gene expression in transformed or infected insect cells. Protein Expr Purif 1996;8:191–203. [PubMed: 8812860]
32. Sorimachi H, Ishiura S, Suzuki K. Structure and physiological function of calpains. Biochem J 1997;328:721–732. [PubMed: 9396712]

33. Carafoli E, Molinari M. Calpain: a protease in search of a function? Biochem Biophys Res Commun 1998;247:193–203. [PubMed: 9642102]

34. Molinari M, Anagli J, Carafoli E. Ca(2+)-activated neutral protease is active in the erythrocyte membrane in its nonautolyzed 80-kDa form. J Biol Chem 1994;269:27992–27995. [PubMed: 7961733]

35. Giguere, Cj; Covington, Md; Schnellmann, Rg. Mitochondrial calpain 10 activity and expression in the kidney of multiple species. Biochem Biophys Res Commun 2008;366:258–262. [PubMed: 18054326]Epub 2007 Dec 2007

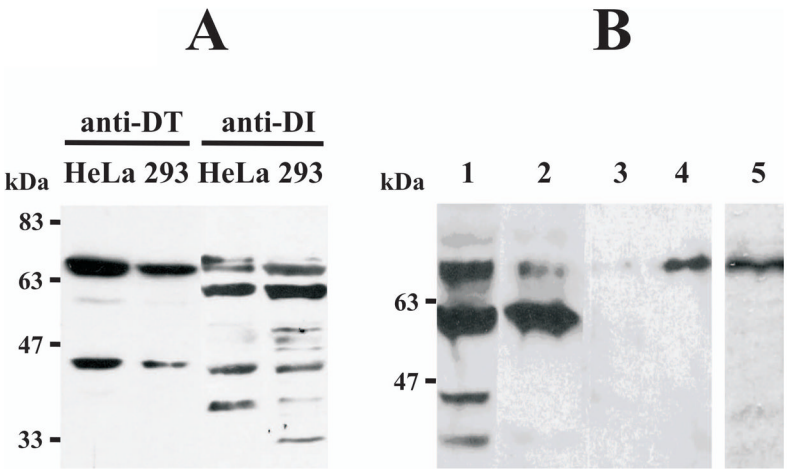

Figure 1.

(A): Western blot analysis of endogenous calpain-10 in HeLa S3 cells using an antibody that targets the N-terminal domain I (anti-DI) or the C-terminal domain T (anti-DT). (B): Purification of endogenous calpain-10a from HeLa S3 cells by immunoprecipitation using anti-DI. The captured calpain-10 on protein A/G agarose beads was eluted with a peptide that was used to raise the primary antibody. Lane 1: direct lysate; lane 2: flowthrough; lane 3: first elution; lane 4: second elution; lane 5: silver staining of the purified calpain-10.

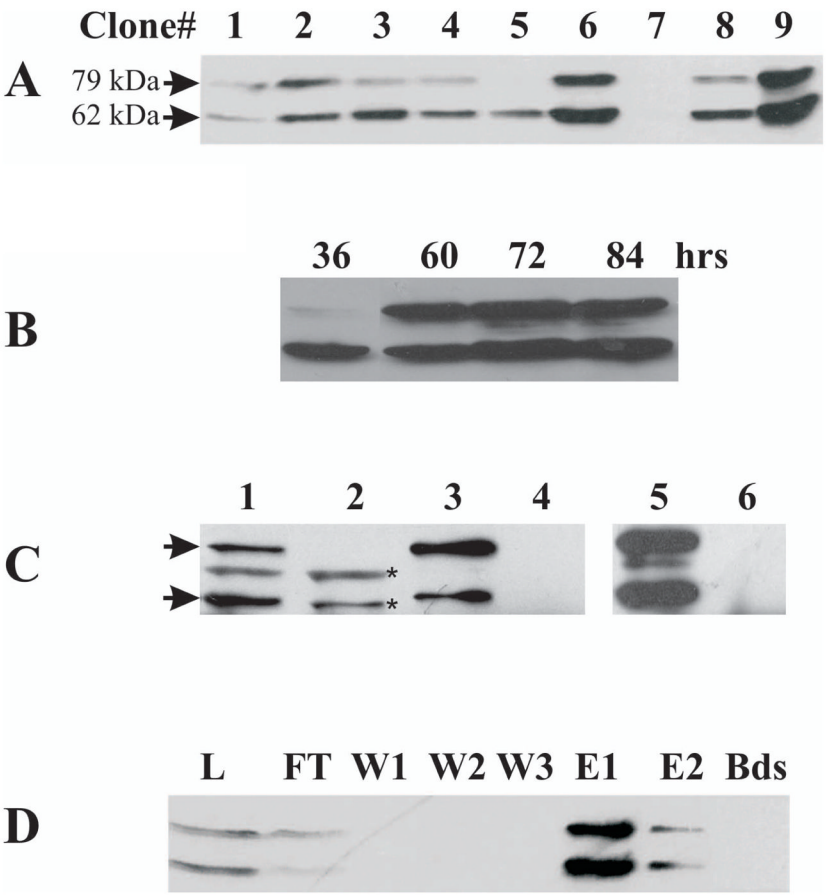

Figure 2.

(A): Western blot analysis of the overexpressed human calpain-10 in different stably transfected 293F cell lines. Anti-V5 antibody was used to probe the expression of recombinant calpain-10 (indicated as arrows). Lanes 1–6 and 8–9 were from different stably transfected 293F cell lines. Lane 7 was from the negative control with an empty vector. Lane 9 was from the 293F-E3 cell line that showed the highest expression level. (B): Time course of the expression of recombinant calpain-10 in 293F cells. The cells were recovered 36, 60, 72, or 84 hrs after transfection, respectively. Western blots were probed with anti-V5 antibody. (C): Western blot analysis of recombinant calpain-10 overexpressed in 293F-E3 cells. Lane 1: unpurified proteins from 293F-E3 cells probed with anti-DI; lane 2: unpurified proteins from untransfected 293F cells probed with anti-DI; lane 3: Ni-NTA purified recombinant calpain-10 from 293F-E3 cells probed with anti-DI; lane 4: Ni-NTA purified proteins from untransfected 293F cells probed with anti-DI; lane 5: Ni-NTA purified recombinant calpain-10 from 293F-E3 cells probed with anti-V5; lane 6: Ni-NTA purified proteins from untransfected 293F cells probed with anti-V5. Recombinant calpain-10 was indicated with an arrow and endogenous calpain-10 was marked with a star. (D): Purification of the recombinant calpain-10 from 293F-E3 cells using Ni-NTA agarose beads. Western blots were probed with anti-V5 antibody. L: direct lysate; FT: flowthrough; W1-W3: washes; E1: the first elution; E2: the second elution; Bds: proteins retained on the agarose beads.

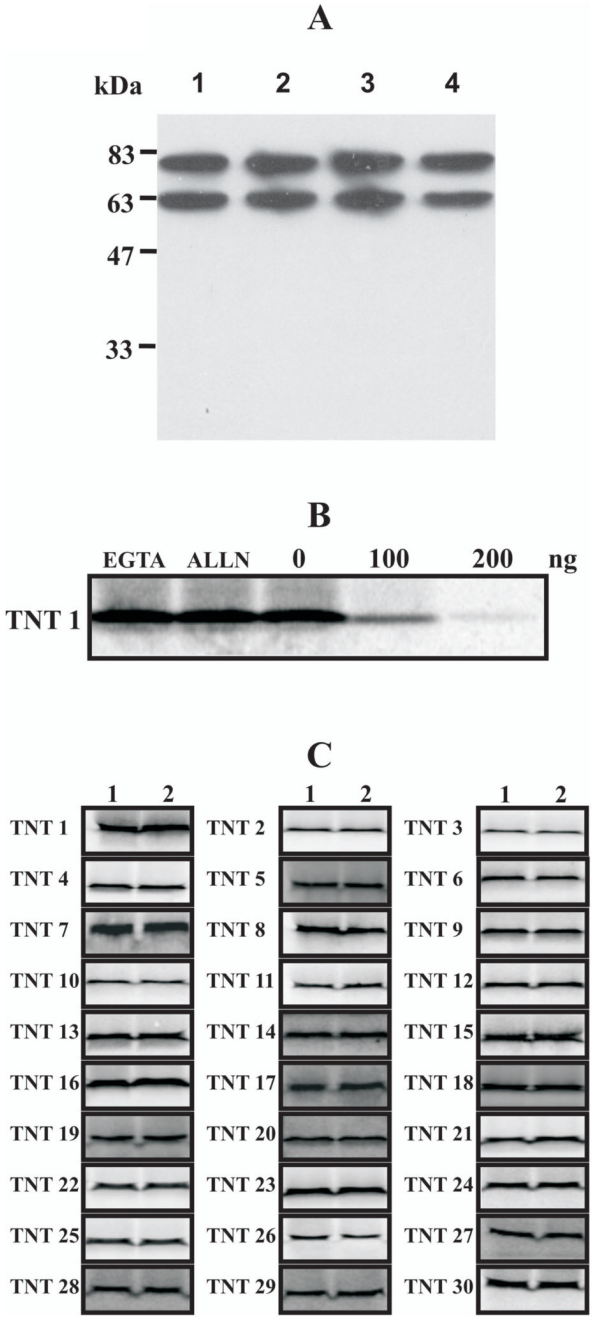

Figure 3.

(A): *In vitro* autolytic analysis of purified calpain-10 overexpressed in 293F-E3 cells. $CaCl_2$ was added to a final concentration of 5 mM to initiate the reaction. EGTA was used to replace $CaCl_2$ in the negative control. Western blot analysis was carried out using anti-V5 antibody. Two different batches of purified calpain-10 were used here. Lanes 1 and 3: purified calpain-10 in an autolysis buffer containing Ca^{2+}; lanes 2 and 4: purified calpain-10 in an autolysis buffer containing EGTA. (B): *In vitro* proteolytic analysis of a radiolabeled short peptide using rat calpain-2. An aliquot of the TNT product was incubated at 30 °C for 2 hr with different amount of purified calpain-2 (0–200 ng) or with 100 ng of calpain-2 in the presence of EGTA or a calpain inhibitor ALLN. A large number of such digestions were performed and only one was

shown here to save space. (C): *In vitro* proteolytic analysis of 30 radiolabeled oligopeptides using calpain-10 purified from stably transfected 293F-E3 cells. An aliquot of each TNT product was incubated with an appropriate amount of Ni-NTA purified calpain-10 at 30 °C for 6 hr. In each panel, lane 1: digestion using Ni-NTA eluate from the lysate of untransfected 293F cells; lane 2: digestion using Ni-NTA purified calpain-10 overexpressed in 293F-E3 cells. A total of 72 oligopeptides were tested and 30 were shown here to save space.

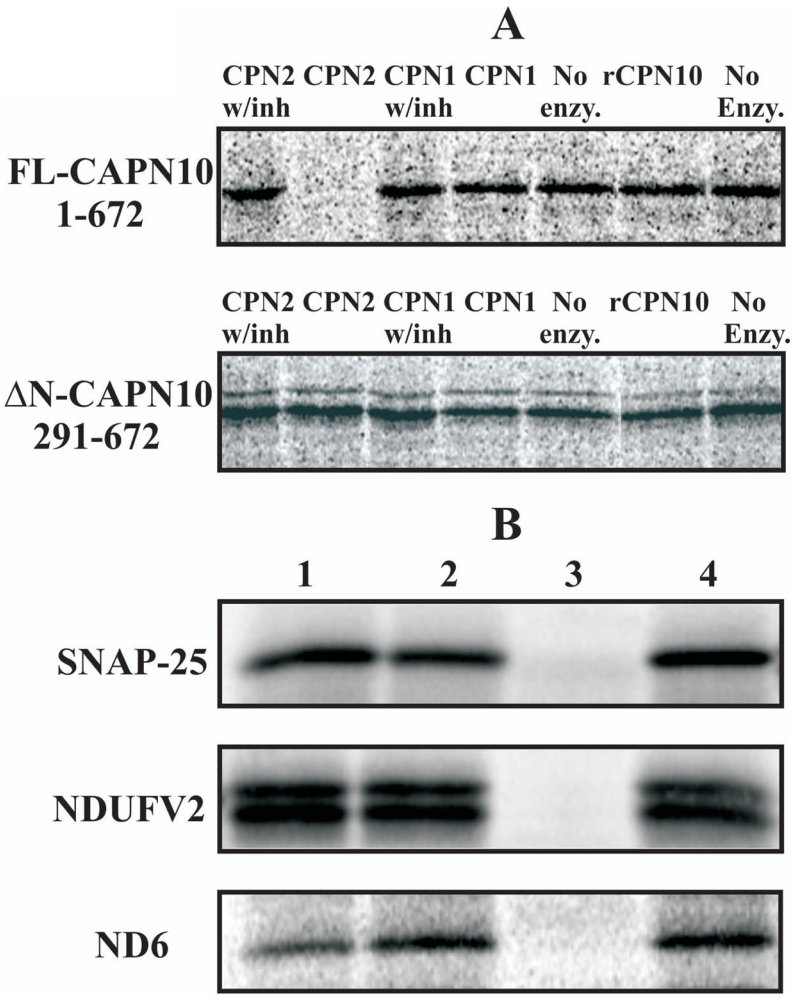

Figure 4.

(A): Proteolytic analysis of radiolabeled calpain-10 using different proteases. [^{35}S]-methionine-labeled full-length calpain-10 (FL-CPN10, residues 1–672) and N-terminal truncated calpain-10 (ΔN-CPN10, residues 291–672) were generated by TNT. Lane 1: with pre-inhibited calpain 2 by ALLN; lane 2: with active calpain-2; lane 3: with pre-inhibited calpain 1 by ALLN; lane 4: with active calpain-1; lane 5: without any protease; lane 6: with purified, recombinant calpain-10; lane 7: without any protease. (B): *In vitro* proteolytic analysis of full-length SNAP-25, NDUFV2, and ND6 using human calpain-10 purified from stably transfected 293F-E3 cells or commercially available rat calpain-2. An aliquot of each TNT product was incubated at 30 °C with approximately 50 ng of calpain-2 for 2 hr or 150–200 ng of purified calpain-10 for 6 hr. In each panel, lane 1: with calpain inhibitor ALLN at 10 μM; lane 2: without calpain; lane 3: with calpain-2; lane 4: with purified calpain-10.