

Purification and Properties of the *Dictyostelium* Calpain-like Protein, Cpl^{†,‡}

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ABSTRACT: Calpains are intracellular, cysteine proteases found in plants, animals, and fungi. There is emerging evidence that they are important mediators of cell adhesion and motility in animal cells. Because the cellular slime mold, *Dictyostelium discoideum*, is a genetically tractable model for cell adhesion and motility, we have investigated whether a calpain-like protein is expressed in this organism. Contig 13130 (Sanger Institute *Dictyostelium* sequencing project) was identified as a three-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 72278 Da cytosolic protein. Weak caseinolytic activity inhibitable by cysteine protease inhibitors was copurified with Cpl immunoreactivity, and purified Cpl appeared to undergo autoproteolysis upon transfer to inhibitor-free buffer. The major cleavage, generating a 51291 Da form, occurred after Pro 189. The Cpl domain structure resembles mammalian calpain 10, comprising an N-terminal catalytic domain followed by tandem calpain D-III domains. The putative catalytic domain appears to possess His and Gln active site residues, instead of the canonical His and Asn residues in calpains. The active site Cys has not yet been identified, and definitive proof of a proteolytic function awaits further study. Its phylogenetic distribution in *D. discoideum* and several protists suggests that the calpain D-III domain evolved early in eukaryotic cells.

Calpains comprise a large family of intracellular, cysteine proteases that function as signal transduction components in a variety of important cellular pathways including sporulation and alkaline adaptation in fungi (1, 2), aleurone cell layer development in plant seeds (3), development of optic lobes in *Drosophila melanogaster* (4), and sex determination in *Caenorhabditis elegans* (5, 6). Among vertebrates, less is known of the specific functions of the calpains. However, inactivating mutations in calpain 3 have been shown to be responsible for limb-girdle muscular dystrophy type 2A (7, 8). Ablation of the mouse gene encoding the small subunit common to the conventional calpains (m- and μ -calpain) is lethal during gestation (9), implying an indispensable function for these calpains at the organismal level. Much recent evidence strongly implies a role for animal calpains in cell motility (10–13) and cell–substratum interactions (14–18).

Major problems impede understanding of calpain function in multicellular animals. The human genome encodes 14 calpains (19). With the exception of m- and μ -calpains, little is known of their function at the protein level, including the substrate selectivity of their presumed proteolytic activities. Therefore, there is great potential for functional redundancy, especially among the calpain genes that are expressed in many different cell types (19). A second problem is the diploid nature of the genome of higher organisms, making classic genetic manipulation a challenging avenue of investigation. Identification of calpain-like proteins in genetically tractable multicellular organisms should facilitate elucidation of their physiologic functions.

Dictyostelids are simple eukaryotic amoebas that have many advantages as a model system for exploring cell cytoskeleton–plasmalemma interactions, cell motility, and development of multicellularity (20). Moreover, they are haploid, and established techniques are available for the generation, selection, and analysis of mutants that would be powerful tools to genetically establish calpain function (20). In this report, we have identified and isolated a calpain-like protein (Cpl) in *Dictyostelium discoideum*. The domain organization of Cpl resembles mammalian calpain 10, including the presence of tandem calpain D-III domains. The latter are C2-like domains that are thought to be important in binding of calpains to phospholipids or other targeting sites in cells (21, 22). Blastp analysis indicated that Cpl is most similar to mouse calpain 10 (E score = 2×10^{-10}). It bears little resemblance to the recently described *Trypanosome* calpain-like genes (23). These studies represent the first step in analysis of Cpl function, which may shed light on the physiologic roles of calpain 10 and similar mammalian calpains.

MATERIALS AND METHODS

Materials. DEAE-Sepharose and phenyl-Sepharose were obtained from Pharmacia. Bio-Gel A-1.5m and electrophoresis supplies were purchased from Bio-Rad. The Ax-3 strain of *D. discoideum* was obtained from ATCC (ATCC no. 28368). Two *Dictyostelium* 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). Polydeoxyribonucleotides were synthesized by Integrated DNA Technologies.

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Dictyostelium Culture. *D. discoideum* strain Ax-3 amoebas were cultured at ambient laboratory temperature (23–25 °C) using standard media and techniques (24).

Hydrodynamic Properties of Cpl. *Dictyostelium* amoebas were lysed by homogenizing in 1 volume of lysis buffer (buffer A containing 150 mM NaCl and 1% NP-40 detergent). After incubation on ice for 60 min, the lysis mixture was passed through a 25-gauge needle and centrifuged at 12000g for 30 min. The supernatant was applied to sucrose gradient ultracentrifugation or gel filtration on a calibrated Sephadex G200 column (25). Cpl 77 kDa immunoreactivity in fractions was determined by immunoblotting. Relative $s_{20,w}$, Stokes radius, and molecular mass were determined as previously described (25). The protein standards used, their sedimentation coefficients, and Stokes radii were as follows: 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 by a previously described method (26).

Purification of Cpl. Ax-3 *D. discoideum* were cultured at ambient temperature in SM medium containing autoclaved *Escherichia coli* (24) to a density of $(5-6) \times 10^6$ amoebas/mL. All purification steps were carried out at cold room temperature (5–8 °C). The standard purification buffer was buffer A: 50 mM imidazole hydrochloride, 200 μ M Na₃VO₄, 6 mM EGTA, 50 μ M leupeptin, 15 μ M pepstatin A, 500 μ M PMSF,¹ and 2 mM dithiothreitol, pH 7.4, measured at room temperature. Ten liters of culture was centrifuged at 1200g for 15 min. The pellet (50–60 mL of packed amoebas, estimated using a calibrated centrifuge tube) was washed twice with 60 mL of development buffer: 5 mM Na₂HPO₄, 5 mM KH₂PO₄, 1 mM CaCl₂, and 2 mM MgCl₂, 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 12000g for 30 min.

The 12000g supernatant was diluted to a nominal NaCl concentration of 50 mM by addition of 2 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 150 mM NaCl. The gel was poured into a chromatography column, packed with 100 mL of buffer A plus 150 mM NaCl, and eluted with buffer A containing 320 mM NaCl, collecting 0.5 mL per fraction.

The Cpl-containing fractions (8 mL) were pooled and concentrated to less than 3 mL by placing in a dialysis tube and exposing to dry poly(ethylene glycol) (MW = 30000). The concentrated sample was applied to an 80 mL Bio-Gel A-1.5m gel filtration column preequilibrated with buffer A containing 100 mM NaCl. The flow rate was approximately 4–6 mL/h, and 2 mL fractions were collected.

Pooled Cpl fractions from the Bio-Gel column (10 mL) were rechromatographed on DEAE-Sepharose and eluted with a 40 mL 0.2–0.5 M NaCl gradient in buffer A. The eluted fractions containing Cpl were adjusted to 0.6 M NaCl and applied to a 3 mL phenyl-Sepharose column in the same buffer. The sample was recycled over the column three times. This step was carried out to remove contaminating proteins

¹ Abbreviations: PMSF, phenylmethanesulfonyl fluoride; TCA, trichloroacetic acid.

Table 1: Purification of Cpl^a

procedure	protein (mg/mL)	volume (mL)	total protein (mg)	units/ mL	total units	units/ mg of protein
DEAE-Sepharose	4.50	8.00	36.00	180	1440	40
Bio-Gel A1.5m	0.95	10.00	9.50	130	1300	137
phenyl-Sepharose, concd fractions	0.35	0.25	0.09	633	158	1810

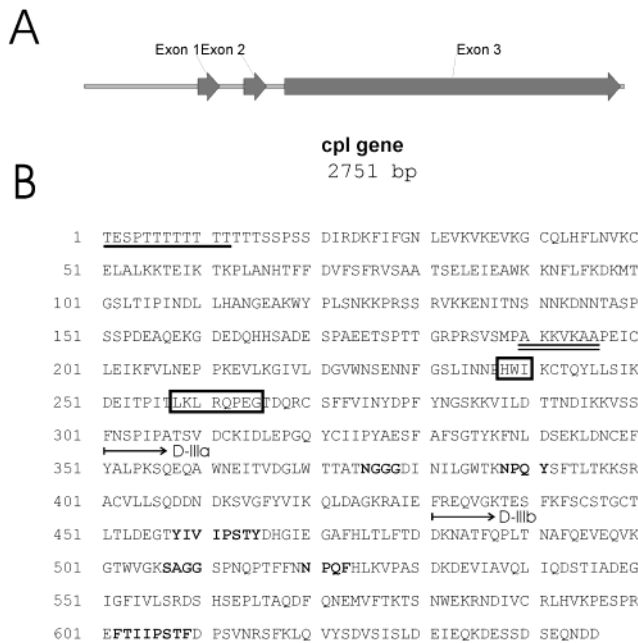
^a The starting material was 10 L of culture containing 5×10^{10} *Dictyostelium* amoebas.

that bound to the phenyl-Sepharose column under these conditions. Cpl did not bind. The 0.6 M NaCl precleared eluate 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, 2–0 M NaCl, in a total volume of 20 mL. Following the gradient, 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 40–60-fold in a Centricon YM-30 centrifugal concentration chamber and stored at 4 °C. The overall yield was 90 μ g of purified Cpl protein from 5×10^{10} amoebas (Table 1).

Protease Assay. Prior to assay, chromatography fractions were centrifuged in Centricon YM-30 membrane devices to remove inhibitors and resuspended in an equal volume of buffer A minus 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 using ¹⁴C-methylated casein (1000 cpm/ μ g) as the substrate (27). Briefly, fractions were incubated with or without 5 mM calcium acetate at 25 °C with 0.5 mg/mL [¹⁴C]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. Ca²⁺-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 minute under the standard assay conditions.

Protein Immunoblotting. Cpl in chromatography fractions was detected by immunoblot analysis using a rabbit immune serum prepared against a sequence (DFQNEMVFTKTSN-WEKRND) 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/nitro blue tetrazolium visual staining (28). In some experiments, blots were scanned for intensity of Cpl staining using a Bio-Rad scanner and Molecular Analyst software.

Cladistic Analysis. D-III domains in proteins were identified by the Smart program (29) and were analyzed for relatedness by the program ClustalW (30) using the neighbor-joining method.



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.) LKLROPEG (110 a.a.) Domain III

FIGURE 1: A calpain-like protein in *D. discoideum*. (A) Gene structure predicted by the fgenesh program. *Dictyostelium* contig 13130 was analyzed, and a single three-exon gene was predicted. (B) Predicted amino acid sequence of Cpl. The entire cDNA sequence was confirmed by reverse transcriptase-PCR of the Cpl mRNA. After purification of Cpl, the amino-terminal sequence was confirmed by direct protein sequencing (bold underlining). A 50 kDa autoproteolysis fragment was found to originate by 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. (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.

RESULTS

Identification of a Calpain-like Protein in *D. 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 code for structures containing the calpain D-III domain. The largest 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 (A. A. Salamov and V. V. Solovyev, unpublished data; CGG WEB server, <http://genomic.sanger.ac.uk/gf/gf.html>) using the yeast gene setting and allowing GC splicing. The predicted 646-residue protein would have a molecular mass of 72278 and contain tandem calpain D-III domains (Figure 1). Blastp analysis (31) indicated closest homology to mouse calpain 10 (E score = 2×10^{-10}).

Analysis of Cpl Expression in *Dictyostelium*. Northern blot analysis of total RNA from *Dictyostelium* amoebas or slugs revealed a single RNA species at approximately 2.4 kb,

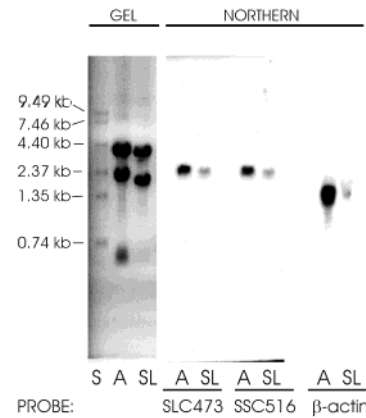


FIGURE 2: Northern analysis of Cpl expression in vegetative amoebas and slugs. *Dictyostelium* were cultured as amoebas in HL-5 medium or allowed to progress to the slug stage following starvation. Total RNA was prepared and subjected to northern blot analysis using 32 P-labeled SLC473 or SSC516 EST clones as the probes. Another set of samples from the same blot was probed with a labeled human β -actin probe. The gel was photographed prior to blotting, and an inverted image of one set of samples is presented on the left of the figure. S = RNA standards; A = amoeba RNA; SL = slug RNA.

Table 2: Cpl Hydrodynamic Properties and Calculated Molecular Mass

Stokes radius	4.3 nm
$S_{20,w}$	4.3 S
partial specific volume	0.734 cm ³ /g
calcd molecular mass	78900 Da
f/f_0	1.46
mass by SDS-PAGE	77000 Da
mass predicted from cDNA sequence	72278 Da

utilizing probes from either of the ESTs obtained from the Tsukuba *Dictyostelium* EST project (Figure 2, middle panel). This is the approximate size expected for a full-length calpain mRNA (32, 33). Although there appeared to be less Cpl mRNA in slugs, there was also much less β -actin detected (Figure 2, right panel). The total RNA preparation appeared not to have been grossly degraded, as the ribosomal RNA bands were intact in both the amoeba and slug samples (Figure 2, left panel).

Purification and Characterization of Cpl. Protein immunoblot analysis employing anti-Cpl antibody was utilized as an analytic tool to explore some of the physical properties of Cpl. Preliminary studies showed that Cpl was easily extracted with neutral buffers from glass-bead-ruptured cells (data not shown), indicating that it is a cytosolic protein. Gel filtration analysis and sucrose density gradient ultracentrifugation of *Dictyostelium* cell lysates indicated that the native Cpl was a slightly asymmetric monomer with a calculated molecular mass of 78900 Da and a frictional coefficient of 1.46 (Table 2). DEAE-Sepharose chromatography of *Dictyostelium* amoeba lysates demonstrated comigration of Ca^{2+} -activated caseinolytic activity with Cpl immunoreactivity, both eluting from the column at about 250 mM NaCl in a linear salt gradient (Figure 3).

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. Bio-Gel chromatography allowed separation of Ca^{2+} -activated case-

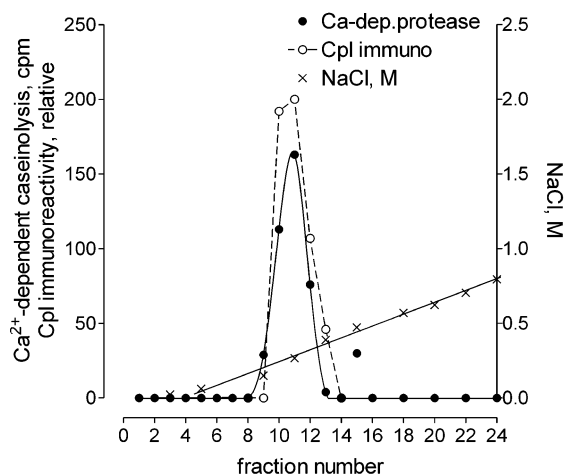


FIGURE 3: DEAE-Sepharose chromatography of Cpl. An amoeba lysate was applied to a DEAE-Sepharose column, and Cpl was eluted with a linear NaCl gradient as described in the Materials and Methods section. Fractions were analyzed for Cpl immunoreactivity (filled circles), calcium-activated caseinolysis (hollow circles), and NaCl concentration (\times).

Table 3: Inhibition of Cpl-Associated Caseolytic Activity by Protease Inhibitors^a

inhibitor	concn (μ M)	% inhibition
PMSF	500	< 10
pepstatin A	100	< 10
leupeptin	50	82
calpeptin	10	80

^a Cpl purified through the Bio-Gel chromatography step was assayed for Ca²⁺-dependent caseolytic activity as described in the Materials and Methods section in the presence of various protease inhibitors.

inolytic activity associated with the Cpl immunoreactivity from an activity that eluted later (not shown). 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 3). Chromatography on phenyl-Sepharose was highly effective in separating Cpl from contaminating proteins (Figure 4). The final purified product had a single major protein band at 77 kDa, which comigrated with the Cpl immunoreactivity (Figure 4B). Trace amounts of 50 kDa protein were also present in this preparation. In several other preparations of Cpl, both 60 and 50 kDa bands were observed.

Autoproteolysis of Cpl. Phenyl-Sepharose-purified Cpl had no detectable caseinolytic activity when assayed by the procedure described in the Materials and Methods section. However, when the preparation was concentrated and pre-incubated in the absence of protease inhibitors for several hours, the 77 kDa band was converted to several 40–50 kDa immunoreactive forms, and caseinolytic activity was generated (Figure 5). These results suggest that autoproteolysis of the 77 kDa form increases Cpl proteolytic activity and is reminiscent of the autoproteolytic activation of mammalian calpains (27, 34). To investigate whether the proteolysis was caused by a contaminant in the phenyl-Sepharose fractions, individual Cpl peak tubes from the phenyl-Sepharose chromatography step were incubated in the absence of inhibitors. The rate of autoproteolysis was estimated by comparison of immunostain densities of the 77, 60, and 50 kDa bands (Figure 6). 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, since a contaminating protease would probably migrate with a different mobility upon gradient elution from phenyl-Sepharose, thereby producing a skewed 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 activity (Figure 7).

To establish the amino-terminal sequence of Cpl and to identify the cleavage site for autoproteolysis, samples of

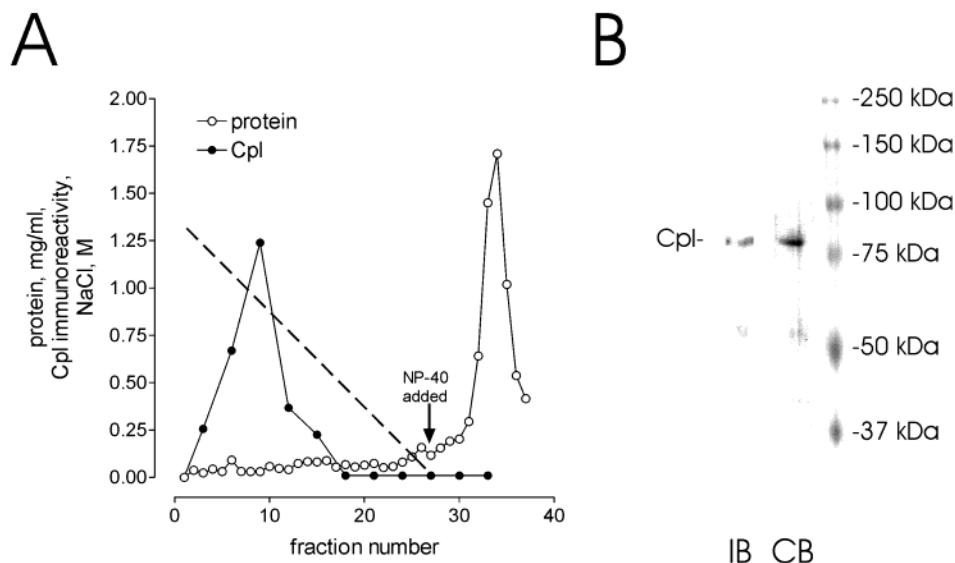


FIGURE 4: Phenyl-Sepharose chromatography of Cpl. Pooled 77 kDa Cpl fractions from the Bio-Gel purification step were applied to a phenyl-Sepharose column and eluted with a decreasing NaCl gradient as described in the Materials and Methods section. Panel A: 77 kDa immunoblot relative density (filled 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 3–9 from the phenyl-Sepharose chromatography were concentrated approximately 20-fold by Centricon filtration. Approximately 200 ng of concentrated samples were subjected to electrophoresis and Coomassie blue staining (CB) or immunoblot analysis for Cpl (IB).

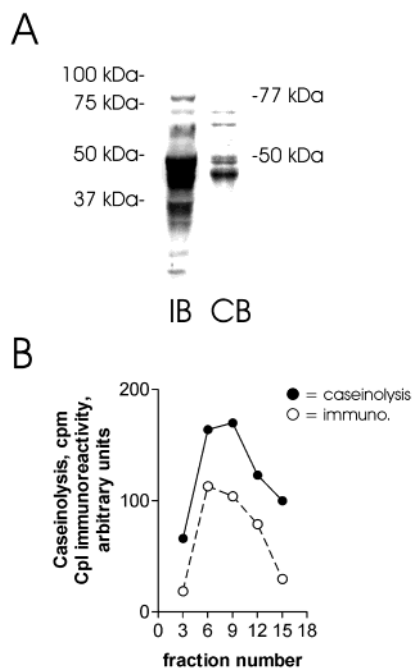


FIGURE 5: Activation of Cpl-associated protease after concentration in the absence of protease inhibitors. Phenyl-Sepharose-purified fractions of Cpl were concentrated as described in the Materials and Methods section. Panel A: Concentrated fraction 9 from the experiment depicted in Figure 4 was analyzed by SDS-PAGE and Coomassie staining (CB) or immunoblotting for Cpl (IB). Panel B: Phenyl-Sepharose fractions representing the Cpl peak were concentrated separately, and the relative density of the 40–50 kDa Cpl immunoreactivity in immunoblots was determined (hollow circles). Caseinolytic activity in the absence of calcium was also measured (filled circles).

native 77 kDa Cpl and the 50 kDa fragment were submitted for amino acid analysis to the Emory University Microchemical Facility. The N-terminal sequence was TESPTTTT-TTTT, as predicted by the fgenesh program. The N-terminus of the 50 kDa fragment was AKKVKAA. Thus, autoproteolytic cleavage occurred after proline 189 (Figure 1) to produce a 51291 Da major fragment.

Phylogenetic Analysis of the Cpl D-III 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*. 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 (Figure 8). 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-IIIs are D-III domains in the plant DEK1-like, Capn 5, and other metazoan calpains.

DISCUSSION

D. discoideum expresses a novel protein that we have named Cpl, for calpain-like protein. Cpl homology to mammalian calpains lies predominantly within its tandem

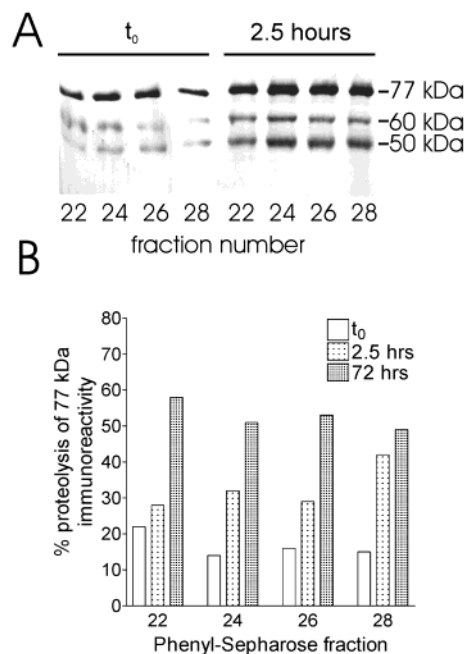


FIGURE 6: Autoproteolysis of purified Cpl. Panel A: Phenyl-Sepharose fractions were concentrated and incubated without protease inhibitors for 2.5 h at 23 °C and then subjected to immunoblot analysis for Cpl. A different Cpl preparation from the one presented in Figure 6 was used, and it contained somewhat more 60 and 50 kDa fragments (panel A, t₀ 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 vs 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 h 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 sum of the densities of the 60 and 50 kDa bands divided by the total staining intensity for all Cpl bands and multiplied by 100.

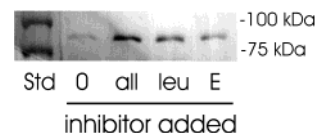


FIGURE 7: Leupeptin and EGTA protect against autoproteolysis of purified Cpl. Pooled concentrated phenyl-Sepharose-purified Cpl was incubated at 23 °C for 4 h in the absence of protease inhibitors (0). Other samples included the inhibitor cocktail used in the isolation buffers (all), 50 μM leupeptin (leu), or 5 mM EGTA (E).

D-III domains. It is important to note that D-III domains have been reported only in calpains to date. 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 (Figure 1). An active site Cys has not been identified. It would have to be different from the calpain DCW consensus sequence (35), which does not appear in Cpl. Nevertheless, we were able to show that a cysteine protease activity is associated with Cpl, 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 gene product (36–38). Cpl has little caseinolytic activity compared with the conventional calpains. Several different preparations

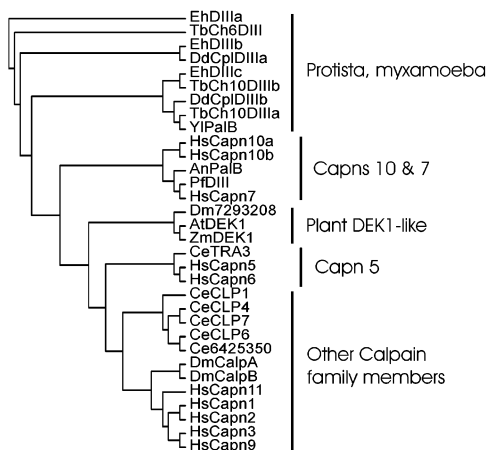


FIGURE 8: Cladogram showing the relatedness of the D-III domains from various organisms. The D-III domains were analyzed for relatedness using the Clustalw program as described in the Materials and Methods section. When D-III domains are present more than once in a sequence, they are lettered a, b, etc., in order starting from the amino terminus. Eh = *E. histolytica*; TbCh6 = *T. brucei* calpain on chromosome 6; Dd = *D. discoideum*; Yl = *Y. lipolytica*; Hs = *Homo sapiens*; An = *Aspergillus nidulans*; Pf = *Plasmodium falciparum*; Dm = *D. melanogaster*; At = *Arabidopsis thaliana*; Zm = *Zea mays*; Ce = *C. elegans*. Abbreviations following the organism name are accepted names for the calpains identified. Numbers following organism names are accession numbers for the nucleotide sequence.

of purified human erythrocyte μ -calpain had turnover numbers of 200–400 μg of casein min^{-1} (nmol of calpain) $^{-1}$ 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. 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 may have a restricted substrate specificity and is thus unable to efficiently cleave model substrates such as casein. This would be predicted for a calpain functioning in a discrete signal transduction pathway, as “bystander” proteolysis would likely be detrimental to overall cell function. It is also possible that Cpl does not function as a protease in *Dictyostelium*. Further studies will be required to address these issues.

Because Cpl does not have a calpain-like catalytic domain, it is not a true calpain family member. However, its general structural and functional properties suggest that it is distantly related to this protease family. The recently described calpain-like proteins in the protist *Trypanosoma brucei*, TbCALP1 and TbCALP2 (23), appear to be substantially different from either Cpl or calpains. Unlike Cpl, their homology to calpains is strictly associated with the catalytic region. The question of their potential proteolytic activity awaits characterization of the expressed proteins. However, because neither TbCALP1 nor TbCALP2 appears to possess active site Cys or His residues (23), it seems unlikely that they will have calpain-like protease active sites.

Phylogenetic comparison of the D-III domains including the new information on protist and *Dictyostelium* sequences (Figure 8) extends the recent study by Sorimachi and Suzuki (39). 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-IIIs with the exception of one Capn 7 orthologue, *Yarrowia 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 (Figure 8). 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.

ACKNOWLEDGMENT

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, is gratefully acknowledged for providing SLC473 and SSC516 EST clones. Where indicated, sequence data were produced by the *D. discoideum* or *E. histolytica* Sequencing Groups at the Sanger Institute and can be obtained from <http://www.sanger.ac.uk/Projects/>. We thank Maura Mericle for providing excellent technical support for these studies.

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