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 euk301(do7yo406(dod269(not)tt785

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database), which contained a three-exon gene, predicted by the fgenesh program (A. A. Salamov and V. V. Solovyev, unpublished data; CGG WEB server, http://genomic.sanger.ac.uk/gf/gf.html) using the yeast gene setting and allowing GC splicing.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, 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+}

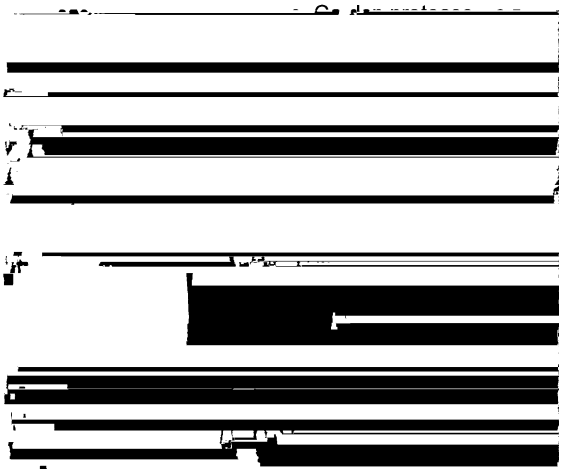

FIGURE 3: DEAE-Sepharose chromatography of Cpl. An amoeba lysate was applied to a DEAE-Sepharose column, and Cpl was eluted with a linear NaCl gradient as described in the Materials and Methods section. Fractions were analyzed for Cpl immunoreactivity (filled circles), calcium-activated caseinolysis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

Table 3: Inhibition of Cpl-Associated Caseinolytic Activity by 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 preincubated 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

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 TESPTTTTTTTT, 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

of purified human erythrocyte μ-calpain had turnover numbers of 200−400 μg of casein min^{-1} (nmol of calpain)$^{-1}$

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