Blastocystis Legumain Is Localized on the Cell Surface, and Specific Inhibition of Its Activity Implicates a Pro-survival Role for the Enzyme*

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Programmed cell death (PCD) is crucial for cellular growth and development in multicellular organisms. Although distinct PCD features have been described for unicellular eukaryotes, homology searches have failed to reveal clear PCD-related orthologues among these organisms. Our previous studies revealed that a surface-reactive monoclonal antibody (mAb) 1D5 could induce multiple PCD pathways in the protozoan Blastocystis. In this study, we identified, by two-dimensional gel electrophoresis and mass spectrometry, the target of mAb 1D5 as a surface-localized legumain, an asparagine endopeptidase that is usually found in lysosomal/acidic compartments of other organisms. Recombinant Blastocystis legumain displayed biphasic pH optima in substrate assays, with peaks at pH 4 and 7.5. Activity of Blastocystis legumain was greatly inhibited by the legumain-specific inhibitor carbobenzyloxy-Ala-Ala-AAsn-epoxycarboxylate ethyl ester (APE-RR) (where AA sn is aza-asparagine) and moderately inhibited by mAb 1D5, cystatin, and caspase-1 inhibitor. Interestingly, inhibition of legumain activity induced PCD in Blastocystis, observed by increased externalization of phosphatidylserine residues and in situ DNA fragmentation. In contrast to plants, in which legumains have been shown to play a pro-death role, legumain appears to display a pro-survival role in Blastocystis.

Programmed cell death in the unicellular protozoa is now accepted as a well established phenomenon. Several stereotypic apoptotic morphological markers similar to those observed in apoptotic metazoan cells have been described in human parasitic protozoa such as Leishmania amazonensis, Leishmania donovani, Trypanosoma cruzi, Trypanosoma brucei, Trypanosoma rhodesiense, Plasmodium falciparum, and Blastocystis (1, 2). Despite a wealth of information on the organelles and cytochemical features involved in protozoan PCD, there is a scarcity of information on PCD-related molecular mediators. Our earlier studies showed that Blastocystis undergoes programmed cell death when exposed to the surface-reactive monoclonal antibody mAb 1D5 with typical features of apoptotic cells (3, 4). mAb 1D5 was shown to target a 30-kDa protein found on the plasma membrane of Blastocystis (5–7). This protein is functionally important, but not all cells within a clonal population would be susceptible to the cytotoxic effects of mAb 1D5 (6). These results suggest that this protein may have multiple localizations and is potentially important for cell survival. In this study, an asparaginyl cysteine protease legumain was identified as the mAb 1D5 targeting protein. Legumain is a recently described lysosomal protease, well conserved and present in plants, mammals, helminth worms, and the protozoan Trichomonas vaginalis (8–12). The active site of legumain contains the catalytic dyad His-Gly-spacer-Ala-Cys, a characteristic shared with caspas es, aspartyl cysteine proteases important as molecular mediators of apoptosis cascades (13). Legumains have specificity for the hydrolysis of bonds on the carboxyl side of asparagines (14) but have also been reported to cleave after aspartate residues, evidenced by their ability to cleave caspase-1 substrates (15, 16). In plants, legumain, also termed vacuolar processing enzyme (VPE), is localized within the plant cell vacuole and is essential for hypersensitive-response cell death, a mechanism of resistance to pathogen infection (15). In mammals, it is predominantly localized in the lysosomes and involved in processing bacterial and endogenous peptides for major histocompatibility complex class II presentation. Legumain is overexpressed in many types of tumors and is associated with enhanced tissue invasion and metastasis. In these cases, the enzyme can also be found on the cell surface, co-localized with integrin β1 (17).

In this study, we report for the first time the detailed characterization of Blastocystis legumain protein as a target of a pre-

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uously described cytotoxic mAb. *Blastocystis* legumain was expressed in *Escherichia coli*, and the purified recombinant protein was used to define enzymatic properties. Cellular localization of this protein was investigated by anti-legumain antibody and mAb 1D5. The involvement of legumain in programmed cell death was investigated by protease inhibitor assays, annexin V, and TUNEL apoptosis assays to determine the extent of phosphatidylserine externalization and DNA fragmentation, respectively. The data strongly suggest that *Blastocystis* legumain has a key role in the regulation of *Blastocystis* cell death.

**EXPERIMENTAL PROCEDURES**

*Parasite Cell Culture—Blastocystis* isolate B (subtype 7) was isolated from the stool of a local patient. Parasite cells were cultured in Iscove's modified Dulbecco's medium containing 10% inactivated horse serum and incubated anaerobically at 37 °C in an Anaerojar (Oxoid). 4-Day-old cells at log phase were used for all experiments. Cell number was counted with a hemocytometer, and their viability was examined morphologically by a light microscope.

**Two-dimensional Electrophoresis—**Parasite lysate was prepared by three freeze-thaw cycles in liquid nitrogen and a 37 °C water bath. Cell debris was removed by centrifugation at 14,500 × g for 10 min at 4 °C. Total proteins from clarified cell lysate were precipitated by 10% trichloroacetic acid. The dried pellet was dissolved in 20 mM Tris-Cl buffer, pH 7.0, and protein concentration was determined by the Bradford assay (Sigma). The first dimension IEFs were performed on 18-cm IEF gel strips (pH 4–7, Bio-Rad) following the manufacturer's instructions. Briefly, the dried strips were rehydrated at room temperature overnight with 375 µl of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 50 mM dithiothreitol, 3% IPG buffer, 0.02% bromphenol blue) and 50 µg of protein. IEF strips were loaded onto a Protein IEF cell (Bio-Rad) by using the following parameters: 500 V for 500 V-h; 1000 V for 500 V-h; 8000 V gradient for 4500 V-h; and 8000 V for 64,000 V-h. Subsequently, strips were soaked in 10 ml of equilibration solution (7 M urea, 0.05 M Tris-Cl, pH 6.8, 30% glycerol, 2% SDS) supplied with 0.1% dithiothreitol for 15 min, removed, and further soaked in equilibration solution containing 0.25% iodoacetamide and 0.02% bromphenol blue for 15 min. Strips were laid on top of a 10% SDS-polyacrylamide gel (Protein II XI, Bio-Rad), and electrophoresis was performed at 5 °C. Current was kept constant at 7 mA per gel for 1 h and then increased to 25 mA per gel for another 6 h. Protein was visualized by Coomassie Brilliant Blue staining.

**Protein Identification—**A gel slice encompassing the protein of interest was cut out, soaked in 200 µl of 50% (v/v) acetonitrile with 50 mM ammonium bicarbonate, and incubated at 37 °C for 30 min to destain. The gel slice was dried for 5 min using a SpeedVac concentrator and soaked in 20 µl of trypsin (3.33 ng µl⁻¹, diluted in 50 mM ammonium bicarbonate) at 30 °C overnight or 37 °C for 4 h. Peptides were extracted using 50% (v/v) acetonitrile with 0.5% (v/v) trifluoroacetic acid (200 µl) followed by sonication (10 min) at 37 °C. The solution was dried using a SpeedVac concentrator and redissolved in 50% (v/v) acetonitrile with 0.5% (v/v) trifluoroacetic acid (1.5 µl). Peptides were desalted using a ZipTip (Millipore) and mixed with an equal volume of matrix solution (α-cyano-4-hydroxy-cinnamic acid (5 mg) dissolved in 50% (v/v) acetonitrile with 0.5% (v/v) trifluoroacetic acid (1 ml)) and spotted (1 µl) onto a MALDI plate (384 Opti-TOF®, Applied Biosystems). After air drying, spots were identified by a combination of mass spectrometry followed by tandem mass spectrometry (10 largest peaks) using an ABI 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems). Peptides derived from trypsin were used as an internal standard. The tandem mass spectrometry results were analyzed by searching the SwissProt Protein Data Base using Mascot version 1.9.

**Western Blotting—**mAb 1D5, a murine IgM monoclonal antibody produced in this laboratory (7), was purified using an affinity column (Affilaid). Anti-human legumain was purchased commercially (Cell Signaling). Parasite cells were lysed in buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 20 mM β-glycerophosphate, 1 mM Na₂VO₄, Roche Applied Science complete protease inhibitor), and the soluble fraction was obtained by centrifugation (14,500 × g, 10 min) at 4 °C. The total protein concentration was determined by the Bradford assay. 50 µg of total protein or 5 µg of purified protein was electrophoresed on a 10% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (GE Healthcare) by semi-dry blotting (Bio-Rad), blocked with 2% bovine serum albumin in TBS-T (triethanolamine-buffered saline with 0.1% Tween), incubated with primary antibody, washed, incubated with secondary antibody, visualized by chemiluminescence (ECL Plus, GE Healthcare), and exposed to an x-ray film (Eastman Kodak Co.).

**Legumain Cloning and Expression—**PCR primers were synthesized according to the peptide sequences from mass spectrometry as follows: P1 (5′-TTTGTNTCNATGNCNTVCN-CGN-3′ and complementary sequence); P4 (5′-GATGACTTTCANCGCNACNTNAAG-3′ and complementary sequence); P6 (5′-TTTAACTCGANCANCGACTNCAGC-3′ and complementary sequence); P7 (5′-TACCTNGAAGAGATGTCVNCTNGN-3′ and complementary sequence), where N represents A, T, G, or C. Forward and reverse primers were picked randomly. In total, 12 primer pair combinations were used for PCR. PCRs were performed with the Faststart high fidelity PCR system (Roche Applied Science) according to the manufacturer’s protocol with *Blastocystis* cDNA library as template. PCR products were electrophoresed in a 1% agarose gel. Appropriate PCR products were excised, purified from the gel, and cloned into pCR2.1 (Invitrogen) by TOPO cloning for sequencing. Five positive plasmids were sequenced with M13⁺ and M13⁻ primers, and consensus sequence was obtained. This product was amplified again by PCR using primers (forward, 5′-TTAGGATCCATGTGGTTGTAGATGCTGCTATT-3′, and reverse, 5′-ATCTCGAGAAGGCGCAGTCCTGCTT-3′). These primers contained BamHI and XhoI restriction sites to allow directional cloning into *E. coli* expression vector pGEX-6p-1 (GE Healthcare). pGEX-6p-1 containing the *Blastocystis* legumain gene was transformed into *E. coli* BL21 (DE3) and amplified in 500 ml of LB containing 100 µg ml⁻¹ ampicillin at 37 °C and 200 rpm. After A₆₀₀ reached 0.6–0.8, isopropyl 1-thio-β-D-
galactopyranoside at a final concentration of 0.5 mM was added. Cells were cultured at 16 °C overnight and then harvested by centrifuging at 5000 \times g for 10 min. The pellets were resuspended in 20 ml of cold PBS and disrupted by sonication. Protein expression was examined using a 10% SDS-polyacrylamide gel.

**Purification of Recombinant Legumain**—The lysate of transformed BL21(DE3) cells was clarified by centrifuging at 25,000 \times g for 20 min at 4 °C, incubated with 2 ml of glutathione-Sepharose 4B slurry (GE Healthcare) at room temperature for 2 h, and washed with 5 volumes of PBS. Legumain was eluted with 2 volumes of elution buffer (10 mM reduced glutathione, PBS). The eluant was concentrated and purity assessed by SDS-PAGE.

**Enzymatic Characterization of Legumain**—Purified recombinant legumain (10 μg) was placed in a 96-well microplate, and the reaction was started by adding the legumain-specific substrate Z-Ala-Ala-Asn-NHMec (10 μM final concentration) in assay buffer (PBS, containing 1 mM dithiothreitol, 1 mM EDTA, and 0.1% CHAPS) and incubated at 30 °C. Fluorescence was determined at 5 and 35 min at excitation 353 nm, emission 442 nm by a TECAN fluorescence plate reader. Rate of change between these two readings was the measurement of protease activity (relative fluorescence units/min).

**Cellular Localization of Blastocystis Legumain**—Cells (1 \times 10^7) were washed with cold PBS and incubated in Iscove’s modified Dulbecco’s medium containing 5 μg of fluorescein diacetate at 37 °C for 10 min, washed, and fixed with 3.7% (v/v) formaldehyde on ice for 30 min. To permeabilize cells, cells were incubated with 0.1% Triton X-100 for 5 min. Cells were incubated with primary antibody (mAb 1D5 or anti-legumain, 1.2 μg ml\(^{-1}\)) in PBS containing 3% bovine serum albumin) for 2 h at room temperature, washed, and incubated with AlexaFluor 594-conjugated secondary antibody (anti-mouse IgM or anti-goat IgG, 2 μg ml\(^{-1}\) in PBS containing 3% bovine serum albumin) for 1 h at room temperature. The cells were washed and stained with 4',6-diamidino-2-phenylindole and viewed under a confocal microscopy (Olympus FV500).

**FIGURE 1. Identification of mAb1D5 target protein.** A, Coomassie Brilliant Blue (CBB)-stained two-dimensional electrophoresis gel of total Blastocystis proteins (left) and Western blot (WB) analysis of total proteins probed with mAb 1D5 (right). A mAb 1D5-reactive spot (arrow) was used to identify the corresponding antigen from a Coomassie Brilliant-stained gel and excised for mass spectrometric identification. B, peptide sequences from mass spectrometry are underlined. PCR was performed with primer combinations, and the appropriate-sized PCR product was sequenced, and the protein sequence was obtained.

**FIGURE 2. Multiple sequence alignment of the catalytic domain of the legumain family.** The sequences containing known or putative catalytic residues in the legumain family were aligned by Vector NTI. Other residues in the catalytic domains were highly conserved in the legumain family. Sequences were compared, and their accession numbers were as follows: mouse (NP_035305), rat (NP_071562), human (AAH03061), bovine (NP_776526), frog (NP_776526), zebra fish (NP_999924), Schistosoma (CAAB71158), rice (BAC41386), tobacco (CAE85998), Blastocystis (ACO24555), Hemonchus (CAJ45481), and Trichomonas (AAQ93040).
Annexin V Apoptosis Assay—Loss of membrane permeability and exposure of phosphatidylserine (PS) were detected using an annexin V-fluorescein isothiocyanate apoptosis detection kit (BioVision) following the manufacturer’s instructions. In brief, 4-h treated (with antibody or inhibitors mentioned previously) or untreated cells (1 × 10^7) were washed with cold PBS and incubated with 0.21 μg ml⁻¹ annexin V-fluorescein isothiocyanate and 2.5 μg ml⁻¹ propidium iodide at room temperature for 10 min. The cells were washed, resuspended in 500 μl of PBS, and analyzed by a flow cytometer (DAKO CyAn ADP) to read green and red fluorescence.

TUNEL Assay for DNA Fragmentation—TUNEL was performed using APO- bromodeoxyuridine kit (Invitrogen) following the manufacturer’s instructions. In brief, 24-h treated (with antibody or the inhibitors mentioned previously) or untreated cells (1 × 10^7) were washed with cold PBS and incubated with 0.21 μg ml⁻¹ annexin V-fluorescein isothiocyanate and 2.5 μg ml⁻¹ propidium iodide on ice for 30 min, washed, and resuspended in 1 ml of 70% cold ethanol at −20 °C for 30 min to permeabilize cells. The cells were washed and incubated with 50 μl of labeling solution at 37 °C for 1 h. After rinsing, cells were stained with AlexaFluor 488-conjugated anti-bromodeoxyuridine antibody at room temperature for 30 min. The cells were washed, resuspended in 500 μl of PBS, and analyzed by a flow cytometer (DAKO CyAn ADP) to read green fluorescence.

RESULTS
Identification of mAb 1D5 Target Protein—Total Blastocystis proteins were separated by two-dimensional electrophoresis and probed with mAb 1D5. A distinct spot of 30 kDa, consistent with our previous studies (6, 7), was observed (Fig. 1A). The corresponding protein spot in the Coomassie Brilliant Blue-stained gel was analyzed by mass spectrometry. In total, seven tryptic peptides were sequenced (Fig. 1B, underlined). Among them, peptides 2, 3, and 5 matched the catalytic domain of legumain sequences from human, rat, mouse, and bovine origins. This suggests that the protein analyzed might be a member of the legumain family. Peptides 1, 4, 6, and 7 did not match any conserved regions of legumain and might be unique to Blastocystis legumain and located at the N or C terminus. Degenerate PCR primers were designed according to the sequences of these seven peptides resulting in 12 pairs of PCR primer combinations. The longest PCR product of ~1 kb was cloned and sequenced. It included 990 bp and encoded a 33-amino acid protein (GenBank™ accession number ACO24555) with a theoretical molecular mass of 37.5 kDa and pI value of 4.9, as calculated by the Vector NTI program. The full-length gene was obtained by 5’-rapid amplification of cDNA ends and 3’-rapid amplification of cDNA ends (1194 bp, supplemental Fig. S1; GenBank™ accession number GU124590). This sequence included a highly conserved catalytic domain that had 40–60% similarity to that of legumains from other origins and by ClustalW was shown to possess unique N- and C-terminal regions (data not shown). As with other legumains, the Blastocystis orthologue presumably codes for a precursor that is auto-cleaved at the C-terminal asparagine residue (18), possibly at Asn-285 to result in the mature form (30-kDa protein). Blastocystis legumain contains a catalytic dyad with the motif His-Gly-spacer-Ala-Cys, which is also found in other legumains (Fig. 2) and in caspases (13). Interestingly, the first 16 amino acids (KVF-SIALLRVLALAAA) include a putative secretory signal peptide, which was also observed in the legumain sequence of the Chinese liver fluke Clonorchis sinensis (19) and not from legumains of other origins.

FIGURE 3. Expression, purification, and verification of Blastocystis legumain. The Blastocystis legumain gene was inserted into pGEX-6p-1 and expressed in E. coli BL21 (DE3) with induction of 0.5 mM isopropyl 1-thio-β-o-galactopyranoside at 16 °C (lanes 1–3). The expressed recombinant legumain was then purified by glutathione affinity column, and purity was assessed by SDS-PAGE (lane 4). Purified legumain was subjected to Western blotting (WB) and probed with anti-GST to confirm the purification (lane 5). Western blots of purified legumain and Blastocystis cell lysate were performed and probed with anti-human legumain and mAb 1D5. A distinct spot of 30 kDa, consistent with the corresponding protein spot in the Coomassie Brilliant Blue-stained gel was analyzed by mass spectrometry. In total, seven tryptic peptides were sequenced (Fig. 1B, underlined). Among them, peptides 2, 3, and 5 matched the catalytic domain of legumain sequences from human, rat, mouse, and bovine origins. This suggests that the protein analyzed might be a member of the legumain family. Peptides 1, 4, 6, and 7 did not match any conserved regions of legumain and might be unique to Blastocystis legumain and located at the N or C terminus. Degenerate PCR primers were designed according to the sequences of these seven peptides resulting in 12 pairs of PCR primer combinations. The longest PCR product of ~1 kb was cloned and sequenced. It included 990 bp and encoded a 33-amino acid protein (GenBank™ accession number ACO24555) with a theoretical molecular mass of 37.5 kDa and pI value of 4.9, as calculated by the Vector NTI program. The full-length gene was obtained by 5’-rapid amplification of cDNA ends and 3’-rapid amplification of cDNA ends (1194 bp, supplemental Fig. S1; GenBank™ accession number GU124590). This sequence included a highly conserved catalytic domain that had 40–60% similarity to that of legumains from other origins and by ClustalW was shown to possess unique N- and C-terminal regions (data not shown). As with other legumains, the Blastocystis orthologue presumably codes for a precursor that is auto-cleaved at the C-terminal asparagine residue (18), possibly at Asn-285 to result in the mature form (30-kDa protein). Blastocystis legumain contains a catalytic dyad with the motif His-Gly-spacer-Ala-Cys, which is also found in other legumains (Fig. 2) and in caspases (13). Interestingly, the first 16 amino acids (KVF-SIALLRVLALAAA) include a putative secretory signal peptide, which was also observed in the legumain sequence of the Chinese liver fluke Clonorchis sinensis (19) and not from legumains of other origins.

FIGURE 4. pH dependence of Blastocystis legumain. Purified r-legumain and fluorogenic substrate Z-Ala-Ala-Asn-NHMec were incubated at 30 °C with pH buffers ranging from pH 3.0 to 8.8. Activity (relative fluorescence units/min (RFU/min)) was plotted against pH values. Values are means ± S.D. (error bars) from three independent experiments.
Expression, Purification, and Verification of Legumain—Blastocystis legumain (truncated form missing 66 amino acids at the 3’ end when compared with the full-length sequence) was expressed in E. coli (Fig. 3). The appearance and accumulation of the overexpressed protein band in E. coli total extracts after 1-h (Fig. 3, lane 2) and 2-h (lane 3) isopropyl 1-thio-β-D-galactopyranoside induction could be detected in comparison with total extracts before induction (lane 1). About 70% overexpressed protein was insoluble, and soluble recombinant protein was purified using the N-terminal GST tag (Fig. 3, lane 4). The GST-tagged legumain was about 65 kDa and its purity was about 95%. The purified recombinant protein was confirmed by anti-GST antibody on the Western blot (Fig. 3, lane 5). To confirm that the purified recombinant protein was legumain and that this was a bona fide target of mAb 1D5, Western blots of the recombinant protein were probed with anti-human legumain (IgG) or mAb 1D5 with Blastocystis cell lysate as a reference and control. Results showed that there were bands at the expected positions (Fig. 3, recombinant legumain, lanes 6 and 8, 65 kDa, and cell lysate, lanes 7 and 9, 30 kDa). The variations in band intensities may be due to mAb 1D5 and anti-human legumain possessing different affinities for precursor and mature forms of legumain. Furthermore, another truncated legumain gene missing 15 amino acids at the 5’ end, which removes a putative secretary signal peptide, was expressed in E. coli using the same induction method. The His$_6$-tagged protein was then purified, and activity and inhibitor assays were performed to study the effect of the signal peptide on enzyme activity. The results (supplemental Fig. S2) showed that legumain with or without signal peptide displayed similar protease activities.

pH Dependence of Legumain Activity—Purified recombinant legumain was incubated at 30 °C with pH buffers in the range of pH 3.0–
Protozoan Surface Legumain Involved in PCD

Our previous studies showed that the cytotoxic surface-reactive mAb 1D5 induced a number of typical PCD features in Blastocystis (3, 21, 22), including cell shrinkage, caspase-like activity, PS externalization, mitochondrial outer membrane permeabilization, and DNA fragmentation. This study reveals that the target of mAb 1D5 is a surface legumain. Comparison of Blastocystis full-length protein sequence with other legumain sequences showed that Blastocystis legumain was 30.1% fluoroacetate ethyl ester (APE-RR) showed over 90% inhibition, and caspase-1 inhibitor Ac-YVAD-CMK showed over 90% inhibition as well. The percentage of annexin V-positive cells in mAb 1D5-treated cells was 47.04% of the population was annexin V-positive in legumain inhibitor-treated cells; 47.17% of the population was TUNEL-positive in mAb 1D5-treated cells; and 47.04% of the population was TUNEL-positive in cystatin-treated cells; and 47.04% of the population was TUNEL-positive in legumain inhibitor-treated cells. The extensive annexin V-positive population seen in mAb 1D5 and legumain inhibitor-treated cells was abrogated when mAb 1D5 or legumain inhibitor was preincubated with purified r-legumain. There was no significant increase of annexin V-positive cells for cells treated with caspase-1 and cathepsin B inhibitor. The percentage of annexin V-positive cells in mAb 1D5-treated cells might be less than the actual value, which can probably be attributed to the blockage of annexin V access to PS by the surrounding antibodies.

DISCUSSION

Our previous studies showed that the cytotoxic surface-reactive mAb 1D5 induced a number of typical PCD features in Blastocystis (3, 21, 22), including cell shrinkage, caspase-like activity, PS externalization, mitochondrial outer membrane permeabilization, and DNA fragmentation. This study reveals that the target of mAb 1D5 is a surface legumain. Comparison of Blastocystis full-length protein sequence with other legumain sequences showed that Blastocystis legumain was 30.1%
Protozoan Surface Legumain Involved in PCD

with the optimum pH at 7.5, similar to what has been reported in *T. vaginalis* (23). It has been shown that parasitic cysteine proteases have a broad pH profile and serve various extra-lysosomal functions as opposed to the narrow pH range of mammalian lysosomal cysteine proteases that primarily exert their activity in the lysosome (27). Interestingly, *Blastocystis* legumain has biphatic pH dependence, with peaks at pH 4.0 and 7.5, a property that has not been seen in legumains from all other origins, and is infrequently observed among enzymes in general. Some individuals with glucose-6-phosphate dehydrogenase deficiency harbor variant glucose-6-phosphate dehydrogenases that display biphatic pH optimum curves (28, 29). In a study on renin pH dependence, it was shown that Ser-84 of human renin contributes to the biphatic pH dependence of the renin-angiotensinogen reaction (30). This biphatic characteristic was specific for human renin, whereas mouse and rat renins exhibited single peaks. It is presently unclear why *Blastocystis* legumain exhibits two peaks of enzyme activity. The localization of the enzyme on the cell surface and in intracellular compartments is consistent with activity peaks at neutral and acidic pH values. We postulate that *Blastocystis* legumain possesses multiple functions and plays important roles at the cell surface and within acidic compartments. Legumains are generally located within lysosomes of mammalian cells and vacuoles of plant cells but have been reported to localize to the cell surface of metastatic tumors (17) and on the microvillar surfaces of helminth intestinal cells (25). Surface legumains were suggested to activate localzymogens that may aid tumor invasion or participate in helminth alimentary digestion of host proteins. *Blastocystis* cysteine proteases are able to cleave human immunoglobulin A (31) and also induce pro-inflammatory responses in host cells (32). The presence of legumain on the parasite surface may similarly function to activate these proteases. The localization of *Blastocystis* legumain to the cell surface is probably promoted by its uncommon secretory signal peptide. Such a signal peptide has only been explicitly described in the legumain of the Chinese liver fluke *C. sinensis* (19), which was found in the excretory-secretory products as a serological antigen.

*Blastocystis* legumain activity was abolished by the legumain-specific inhibitor APE-RR, an aza-Asn derivative that does not

![FIGURE 7. Annexin V apoptosis assay for inhibitor-pretreated cells.](image_url)
cross-react with caspases, papain, and cathepsin (33). It is moderately inhibited by cystatin and mAb 1D5 and weakly inhibited by the caspase-1 inhibitor Ac-YVAD-CMK. Cystatins are potent inhibitors of the papain-like cysteine peptidases in the unrelated family C1 but also inhibit legumain, due to a separate site on the cystatin molecule (34), although plant and mammalian legumains have been shown to cleave caspase-1 substrates (15, 23). Hence, Blastocystis legumain shares a number of characteristics with legumains of other origins.

In plants, legumain is essential for hypersensitive-response cell death (15) and is also involved in fungal toxin FB1-induced plant cell death (16). Virus-induced gene silencing of VPE revealed loss of VPE and caspase-1 cleavage activities concomitant with loss of PCD features upon tobacco mosaic virus induction, resulting in increased virus proliferation. Hence, VPE-mediated cell death is important for resistance to pathogen infection. VPE targets that mediate hypersensitive-response cell death are currently unknown (35). In contrast to the plant model, our present studies suggest that Blastocystis legumain mediates pro-survival functions. This is evidenced by the significant PS externalization and in situ DNA fragmentation upon incubation with legumain inhibitors. There was a positive correlation between the extent of these PCD features with the degree of legumain inhibition (Fig. 9), with legumain-specific inhibitor and cystatin inducing the greatest amount of PS flipping and DNA fragmentation. The data indicate that PCD in Blastocystis is regulated by surface legumain activity and suggests a pro-survival role for the surface molecule. However, although cystatin only caused 45% inhibition of Blastocystis legumain activity as compared with 90% inhibitory effect by the legumain-specific inhibitor, the extent of cell death induced by cystatin was even higher than that induced by the legumain-specific inhibitor. Because cystatin is also a high affinity inhibitor of family C1 cysteine proteases, it is possible that there is a papain-like cysteine protease downstream of legumain whose inhibition triggers PCD, and by inhibiting both legumain and this hypothetical substrate protease, cystatin causes massive cell death in Blastocystis. Because cystatin is a cell-impermeable inhibitor (36), it is thus unlikely that PCD induction was due to inhibition of some other intracellular enzyme. We speculate that Blastocystis cell surface legumain may be responsible for processing and activating a downstream cysteine protease, which also localizes at the cell surface and has important function in nutrition uptake. Alternatively, cystatin may independently trigger PCD by inactivating papain-like proteases on the parasite surface. Our previous studies showed
Protozoan Surface Legumain Involved in PCD

that mAb 1D5 exposure induces PCD features rapidly, as evidenced by PS externalization and caspase-like activity by 2 h post-induction (4, 22), suggesting a direct role for legumain in Blastocystis PCD. In this study, it was demonstrated that mAb 1D5 was able to inhibit Blastocystis legumain. It is known that enzymes can be inhibited by some of their specific antibodies through mechanisms such as steric hindrance and conformational changes (37). mAbs of different Ig subtypes have been used to study the role of individual cytochrome P450 isoforms. Because of their high specificity and high inhibitory activity, carefully selected mAbs were suggested to be superior to chemical inhibitors to target specific cytochrome P450 enzymes (37). mAb 1D5 is the first reported legumain-specific antibody that has an inhibitory effect on this enzyme. The mechanism by which Blastocystis legumain modulates PCD is presently unclear. Human embryonic kidney 293 cells overexpressing legumain appear more resistant to apoptosis compared with cells that have basal levels and is associated with tumor invasion and metastasis in vivo (17). More work needs to be done to unravel the pro-survival mechanism of Blastocystis legumain and to determine whether it is similar to that of tumor cells. Identification of the substrates of legumain will take us one step further in delineating the legumain-mediated PCD pathway.

This study is the first to provide detailed functional characterization of an unusual protozoan legumain. Blastocystis legumain is localized on the cell surface, has biphasic pH optima, and is associated with PCD. As there have only been a handful of protozoan genes shown to be involved in PCD, the characterization of a surface protease with a possible pro-survival function sheds new light on the mechanisms of PCD among the unicellular eukaryotes.

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