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The Activity of a Developmentally Regulated Cysteine Proteinase Is Required for Cyst Wall Formation in the Primitive Eukaryote *Giardia lamblia**[S]

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Giardia **is an intestinal parasite that belongs to the earliest diverging branch of the eukaryotic lineage of descent.** ***Giardia*** **undergoes adaptation for survival outside the host's intestine by differentiating into infective cysts. Encystation involves the synthesis and transport of cyst wall constituents to the plasma membrane for release and extracellular organization. Nevertheless, little is known about the molecular events related to cyst wall biogenesis in** ***Giardia*****. Among the components of the cyst wall there are two proteins that we have previously identified and characterized: CWP1 (26 kDa) and CWP2 (39 kDa). Expression of these proteins is coordinately induced, and both concentrated within encystation-specific secretory vesicles before their extracellular polymerization. Although highly similar to each other at the amino terminus, CWP2 includes a COOH-terminal 121-amino acid extension. Here, we show that this extension, rich in basic residues, is cleaved from CWP2 before cyst wall formation by an intracellular cysteine proteinase activity, which is induced during encystation like CWPs. Specific inhibitors prevent release of cyst wall materials, abolishing cyst wall formation. We also report the purification, cloning, and characterization of the encystation-specific cysteine proteinase responsible for the proteolytic processing of CWP2, which is homologue to lysosomal cathepsin C. Encystation-specific cysteine proteinase ESCP possesses unique characteristics compared with cathepsins from higher eukaryotes, such as a transmembrane domain and a short cytoplasmic tail. These features make this enzyme the most divergent cathepsin C identified to date and provide new insights regarding cyst wall formation in** ***Giardia*****.**

Giardia, a flagellated protozoan parasite that resides in the intestine of different vertebrate hosts, is the most frequent cause of defined waterborne diarrhea worldwide (1). Infection begins with the ingestion of cysts, followed by their excystation and colonization of the small intestine by the trophozoites (2, 3). To survive outside the hosts intestine, trophozoites differentiate into infective cysts, which are excreted in the feces (2–5). *Giardia* is also an excellent model organism to study the evolution of fundamental cellular processes since it belongs to one of the earliest branches of the eukaryotic lineage of descent (6, 7).

Cyst formation in *Giardia*, also called encystation, involves three well coordinated steps (3–5). Soon after *Giardia* trophozoites are starved for cholesterol (8), encystation progresses through: 1) the expression of encystation-specific genes, such as those necessary for the synthesis and processing of cyst wall components (9–13); 2) the biogenesis of secretory organelles absent in non-encysting trophozoites, the Golgi apparatus (14), and secretory granules (ESVs)[1] (9, 10, 15, 16); and 3) the release of secretory granule's content and assembly of the extracellular cyst wall (17, 18).

Biochemical analyses indicated that the cyst wall consists of both carbohydrate (19, 20) and protein components (9, 10). We have previously identified and characterized two *Giardia* cyst wall proteins, CWP1 and CWP2. *CWP* predict acidic and leucine-rich proteins of 26 (CWP1) and 39 (CWP2) kDa targeted to the secretory pathway by amino-terminal signal peptides. The amino-terminal overlapping domains of CWP1 and CWP2 are 61% identical in sequence; contain a cysteine-rich region and five tandem copies of leucine-rich repeats. Besides these similarities, CWP2 is distinguished from CWP1 by a 121-residue carboxyl-terminal extension that is rich in basic amino acids. This extension, which accounts for the differences in molecular mass and pI of the two proteins, appears implicated in the sorting of the CWP1-CWP2 complex to the ESVs (4, 10).

Interestingly, in purified ESVs CWP2 is mainly found as a 39-kDa protein (26 kDa from the CWP1-like region plus 13 kDa from the alkaline tail); however, in purified cyst walls, only a 26-kDa fragment could be found (4, 10), suggesting that proteolytic processing of CWP2 occurs before this protein is incorporated into the cyst wall.

Formation of cell walls (*i.e.* during cyst or spore develop-

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[1] The abbreviations are: ESV, encystation-specific secretory vesicles; CWP, cyst wall protein; rCWP, recombinant CWP; mAb, monoclonal antibody; ESCP, encystation-specific cysteine protease; DPP IV, *Giardia* dipeptidyl peptidase IV; DTT, dithiothreitol; E64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; PV, peripheral vesicles.

ment) is a common characteristic of several protozoan parasites (3, 4). Cyst wall formation in *Giardia* is an interesting biological problem because the synthesis of cyst wall components takes place within the cell, but their deposit and fibrillar organization occur extracellularly. In consequence, there must be particular mechanisms to carry the precursors and the machinery for cyst wall assembly to the cell surface, avoiding their polymerization inside the cell (4). The purpose of the present study was to analyze the proteolytic processing of CWP2 in the context of the regulated secretory pathway that develops during *Giardia* differentiation into cysts.

Our results indicate that an intracellular cysteine proteinase activity is involved in CWP2 cleavage and that specific inhibitors of this enzymes block the release of cyst wall precursors from the ESVs, abolishing cyst wall formation during differentiation. Due to the importance of this proteinase for the completion of the life cycle of *Giardia*, we attempted its isolation and molecular characterization. The identified gene encodes a protein homologous to lysosomal cathepsins C of higher eukaryotes but, in contrast to those enzymes, *Giardia* cysteine proteinase possesses a transmembrane domain and a short cytosolic tail.

EXPERIMENTAL PROCEDURES

Giardia lamblia Cultivation and Encystation in Vitro—Trophozoites of the isolate WB, clone 1267 (21), were cultured as described (22). Encystation of trophozoite monolayers was accomplished by the method described by Boucher and Gillin (23).

Assay of Proteinase Effects on Encystation in Vitro—Trophozoites were grown in pre-encystation medium for 2 days, the medium was then replaced with encystation medium containing different proteinase inhibitors (see Table I), and cells maintained at 37 °C for 24 h. Cysts were recovered from the medium and trophozoites collected and resuspended in tap water (23). Mature cysts were counted in a Coulter Z1 Cell Counter.

Effect of Protein Transport Inhibitors—Several inhibitors of protein secretion (24–27) (see Table II) were added to encysting medium as described for the proteinase inhibitors. Then, the production of cysts and the pattern of CWP2 by immunoblotting were determined. Controls with solvent only and careful titrations of inhibitors were performed to avoid deleterious effects on other cellular functions.

Immunoblotting and Immunofluorescence Analysis—Western blot was performed as previously reported (10). Filters were blocked and then incubated with monoclonal antibody (mAb) 7D2, specific for CWP2 (10). For immunofluorescence assays, cells cultured in growth medium, pre-encystation medium, or encystation medium were harvested and processed as described previously (10). The specimens were mounted, viewed on a Leica IRME fluorescence microscope; images were captured with a Leica DC250 camera, and processed with Leica QFluoro Software.

Determination of "In Gel" Proteinase Activity—Detection of proteinase activities was done using non-boiled samples in 15% acrylamide gels containing 0.2% gelatin (28). Proteinase activity was assessed by adding inhibitors to the gel incubation buffer. Since substrate-copolymerized acrylamide gels may change the mobility of proteins, the correct molecular weight of the protease activities was determined as following. After standard SDS-PAGE using non-boiled samples, gels were placed over a glass plate and an indicator gel (containing 15% polyacrylamide plus 0.1% recombinant CWP2 or gelatin) was placed on top. A second glass plate was laid over, and the resulting sandwich held in place by two clamps and immersed in a chamber filled with citrate-phosphate buffer, pH 6.0, at room temperature. Diffusion of proteins from the electrophoresis gel to the indicator gel was allowed for 15 h. Once transfer was completed, the indicator gel was immersed into 2% Triton X-100, and incubated for 1 h with gentle shaking at room temperature. Then, it was rinsed twice with distilled water and incubated in citrate-phosphate buffer, pH 6.0, 1 mM DTT, for 6 h at 37 °C. The indicator gels were stained, destained, and interpreted for proteinase activity (28).

Enzyme Purification—To purify the cysteine proteinase, trophozoites were induced to encyst and harvested as described (23). Cell pellet was suspended in 10 mM Tris-HCl buffer, pH 7.6, 0.1% Triton X-100 and sonicated until no intact trophozoites were visualized by light microscopy. The homogenate was centrifuged at 5,000 × *g* for 30 min at 4 °C. To the supernatant, $(NH_4)_2SO_4$ was added at different percentage of saturation at 0 °C. The fraction at 50% saturation was centrifuged and the pellet was dissolved in 25 mM Tris-HCl buffer, pH 8.0. This material was dialyzed for 12 h against 3,000 volumes of 10 mM Tris-HCl buffer, pH 7.6, and applied to an anion-exchange column (NITRAP Q, Bio-Rad). Proteins were eluted using a NaCl continuous gradient (0 to 1 M) and fractions of 1 ml were collected and assayed for proteinase activity using gelatin-containing gels. The active fractions were pooled, concentrated, dissolved in 25 mM Tris-HCl, pH 8.0, and then applied to a Sephadex G-100 column. Finally, affinity chromatography using cystatin-Sepharose was performed as reported (29).

Protein Microsequencing—Purified enzyme was transferred to Immobilon (Millipore) membranes and stained with Coomassie Brilliant Blue R-250. The band was excised and subjected to Edman degradation on a PerkinElmer Biosystems Model 477A Gas-Phase Protein Sequencer as a service of the Protein and Nucleic Acid Shared Facility, Medical College of Wisconsin.

Expression and Purification of Recombinant CWPs (rCWPs)—The cDNA fragments encoding CWP1 or CWP2 genes, without the signal peptide sequence, were inserted into the *Eco*RI/*BamHI* sites of pGEX-4T. The resulting constructs encoding a glutathione *S*-transferase fusion protein were transformed into *Escherichia coli* strain M15 K12. Since preliminary experiments indicated that rCWP2 underwent proteolysis under the conditions used for purification, a His_6 tag was inserted at the 3′-end of the GST-CWP2 construct to perform a two-step purification approach. Purification was done using the GST Gene Fusion System (Amersham Biosciences, Inc.). Once the column with bound protein was washed, the fusion protein was eluted by the addition of 10 mM reduced gluthatione in 50 mM Tris-HCl, pH 8.0. Glutathione *S*-transferase was removed using 10 units of thrombin. For CWP2, we performed a second step purification using the QIAexpressionist System (Qiagen). The sample obtained after been treated with thrombin was mixed with Ni-NTA matrix, washed several times, and eluted by competition with imidazole.

Substrate Assay—Purified rCWP2 was mixed with the purified cysteine proteinase and incubated in citrate-phosphate buffer, pH 6.0, 1 mM DTT for 12 h at 37 °C. The sample was incubated in sample buffer with β-mercaptoethanol, fractionated in 15% SDS-PAGE, and detected by immunoblotting as indicated above.

Phase Separation of Integral Membrane Proteins in Triton X-114 Solution—Encysting trophozoites were suspended in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1.0% Triton X-114 at 0 °C and processed as reported (30). After phase separation, Triton X-114 and buffer were added to the aqueous and detergent phases, respectively, to obtain equal volumes and approximately the same salt and surfactant content for both samples.

Nucleic Acid Analysis—Giardia RNA was extracted from trophozoites by RNA Total Isolation Kit (Promega). Genomic DNA was obtained as described (31). For reverse transcriptase-PCR, cDNA synthesis was performed using total RNA extracted from encysting trophozoites by priming the template with oligo$(dT)_{20}$. NH_2-terminal amino acid sequencing of the purified mature proteinase, in conjunction with codon usage knowledge in *Giardia* (32, 33), allowed the design of a moderately degenerate primer (5′-GA(C/T)GG(A/C/G)AT(T/C)GG(A/C/G)AA(T/C)-GC(A/C/G)(T/A)(C/G)(C/A/G)CA(G/A)GC(A/C/G)TG(T/C)CT(G/C)-3′) encoding the amino acid sequence DGIGNASQACL. For PCR, this guessmer was used together with the oligo$(dT)_{20}$ primer. The PCR product was electrophoresed, gel purified, and radiolabeled. 5′-Rapid amplification of cDNA ends was performed using a commercial kit from Invitrogen and the primers 5′-CAGGGATTACAACATTTCG-3′ and 5′-TCGTTCTTCTTAACTGG-3′. A *G. lamblia* cDNA library in λgt22a was screened as reported (9, 10). Duplicate plate lifts of the library were probed at 60 °C with the ^{32}P-labeled PCR product obtained by reverse transcriptase-PCR. Filters from both screenings were washed twice at intermediate stringency (2 × SSC, 0.1% SDS, at 60 °C). Membranes were then exposed to Kodak X-AR film at −70 °C, and plaques further purified to homogeneity using the same procedure. A λZAP gDNA library screening was performed as described (10). DNA fragments were cloned into pBlueScript $SKII^+$ and submitted for automatic sequencing at the Sequencing Facility, BioResources Center, Cornell University, Ithaca, NY.

Analysis of DNA sequences was performed with the computer program DNAStar (Lasergene). Signal sequence prediction was done using SignalIP (34). SMART (35) was used for searching protein domains and patterns. Homology searches and other structural predictions were performed with software available at ExPASy (www.expasy.ch).

Fragments used as probes were purified from agarose gels and radiolabeled by random primer method (31). For Southern blot analysis,

TABLE I

Effect of protease inhibitors on cyst wall formation and CWP2 processing during Giardia encystation

Trophozoites of *G. lamblia* were induced to encyst in presence or absence of different protease inhibitors. After 24 h, encystation medium was decanted and the cysts counted and subjected to electrophoresis and Western blotting. Results for cyst production are shown as the percentage of cyst generated compared to control without inhibitor and represent the mean of five independent experiments. Values for Western blot determination of relative mobility are in kDa.

Protease inhibitor	Specificity	Membrane permeability	Cyst production	Molecular weight of CWP2
None			100	39 and 26
Antipain (50 μg/ml)	Papain and trypsin	Permeant	47	39 and 26
Pepstatin (1 μg/ml)	Aspartate proteases	May be permeant	98	39 and 26
Chymostatin (60 μg/ml)	Chymotrypsin	Non-permeant	99	39 and 26
APMSF[f] (10 μg/ml)	Serine proteases	Permeant	99	39 and 26
Aprotitin (2 μg/ml)	Serine proteases	Non-permeant	100	39 and 26
Trypsin inhibitor (100 μg/ml)	Serine proteases	Non-permeant	100	39 and 26
Phenylmethylsulfonyl fluoride (170 μg/ml)	Serine proteases	May be permeant	99	39 and 26
TLCK[a] (50 μg/ml)	Serine and cysteine proteases	Permeant	89	39 and 26
TPCK[b] (100 μg/ml)	Serine and cysteine proteases	Permeant	95	39 and 26
Leupeptin (5 μg/ml)	Cysteine proteases	May be permeant	99	39 and 26
EGTA (1000 μM)	Metalloproteases	Permeant	5	39 and 26
ALLN[c] (17 μg/ml)	Cysteine proteases	Permeant	0	39
ALLM[d] (10 μg/ml)	Cysteine proteases	Permeant	0	39
E64[e] (10 μg/ml)	Cysteine proteases	Non-permeant	99	39 and 26
E64c (10 μg/ml)	Cysteine proteases	Permeant	3	39
E64d (10 μg/ml)	Cysteine proteases	Permeant	0	39
Bestatin (50 μg/ml)	Leucine amino peptidases	Permeant	0	No expression

[a] TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone.
[b] TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.
[c] acetyl leucyl leucyl norleucinal.
[d] acetyl leucyl leucyl methionunal.
[e] trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane.
[f] 4-amidino methylsulfonyl fluoride.

10 μg of *G. lamblia* gDNA was digested with several restriction enzymes and electrophoresed in 1% agarose gel. DNA was transferred onto Hybond-N membranes (Amersham Bioscience, Inc.) (31). For Northern blotting, total RNA (10 μg/lane), isolated from trophozoites undergoing encystation for different periods (9, 10, 24), was electrophoresed on 2.2 M formaldehyde, 1% agarose gel and transferred onto Hybond-N membranes. Filters were hybridized as described (10) with the [^{32}P]dATP-labeled full-length cysteine proteinase gene. Final washings were done at 60 °C with 0.1 × SSC and 0.1% SDS.

Overexpression of ESCP in Encysting and Non-encysting Trophozoites—The plasmid PTUBH7Pac (36) was previously modified to introduce the entire ESCP coding region and the influenza hemagglutinin epitope just before the TAA stop codon.[2] Transient transfection of *G. lamblia* trophozoites was done by electroporation as previously described (36–38). Indirect immunofluorescence assays using an anti-hemagglutinin mAb (Sigma) was performed on non-encysting and 24 h encysting trophozoites as described above. LysoSensor™ Green DND-189 (Molecular Probes) was used to label PVs.

RESULTS

Proteinases Involved in Giardia Encystation—To study the participation of proteolytic activities during *Giardia* trophozoites differentiation into cysts, several proteinase inhibitors were added to encystation medium (Table I). Subsequently, the percent of cysts formed and molecular weight of CWP2 in trophozoites were analyzed and compared with controls without inhibitors. When inhibitors of serine proteinases, carboxypeptidases (permeant or not to cell membranes), or non-permeant cysteine proteinases inhibitors were added to encystation medium, cyst formation was unaffected and the molecular weight of CWP2 in Western blots was 39,000 and 26,000 (Table I and Fig. 1*A*), indicating that the proteolytic processing of CWP2 took place. In contrast, permeant cysteine proteinases inhibitors prevented cyst formation and proteolytic processing of this protein. In the presence of EGTA, proteolysis occurred but no cyst formation was observed, suggesting that this drug might block exocytosis of ESVs (Table I).

Immunofluorescence analysis of trophozoites induced to encyst in the presence of permeant cysteine proteinase inhibitors E64d (39) showed that CWP2 was within ESVs (Fig. 1, *B* and *C*), but these vesicles did not release their contents to the cell exterior (Fig. 1*C*). These results suggest that proteolytic processing of the alkaline tail of CWP2 is an indispensable requirement for its release and incorporation into the cyst wall, and that an intracellular cysteine proteinase is directly involved in the process. Under the conditions used in this study, neither cell viability, cell proliferation, nor flagellar motility changed during the time in which the experiments were done.

When the aminopeptidase inhibitor bestatin (40) was used, not only was cyst formation blocked but also the expression of CWPs was abolished. Bestatin's effect on *Giardia* encystation strongly suggests that a specific proteolytic event driven by an aminopeptidase IV (DPP IV) is involved in earlier steps of encystation.[2]

Identification of the G. lamblia Encystation-specific Cysteine Proteinase—Total proteins from *G. lamblia* trophozoites either induced or not induced to encyst were separated using gelatin-containing gels and the presence of proteolytic activities was analyzed. Gels were incubated for 15 h in citrate/phosphate buffer, pH 6.0, at 37 °C in the presence of 1 mM DTT. Results showed that multiple bands with gelatinolytic activity were present in both non-encysting trophozoites and those induced to encyst. However, one protease of ~45 kDa was strongly induced during encystation (Fig. 2*A*). When we analyzed the increase of proteolytic activity of this proteinase, we not only noticed the almost identical kinetics of induction compared with that of CWP2 but also that its pattern of expression agreed with the proteolytic processing of that protein (Fig. 2*B*). It is worth mentioning that the number of gelatinolytic bands was always dependent on the percentage of acrylamide of the gel, the incubation time, and the quantity of protein loaded onto the gel. For those reasons, careful optimization of the experiments was necessary. When proteinase inhibitors such as E64 (inhibitor of cysteine proteinases) and leupeptin (inhibitor of serine and cysteine proteinase) were added in the gel incubation buffer, the gelatinolytic activity of ~45 kDa was

[2] M. C. Touz, M. J. Nores, I. Slavin, L. Piacenza, D. Acosta, C. Carmona, and H. D. Luján, unpublished data.

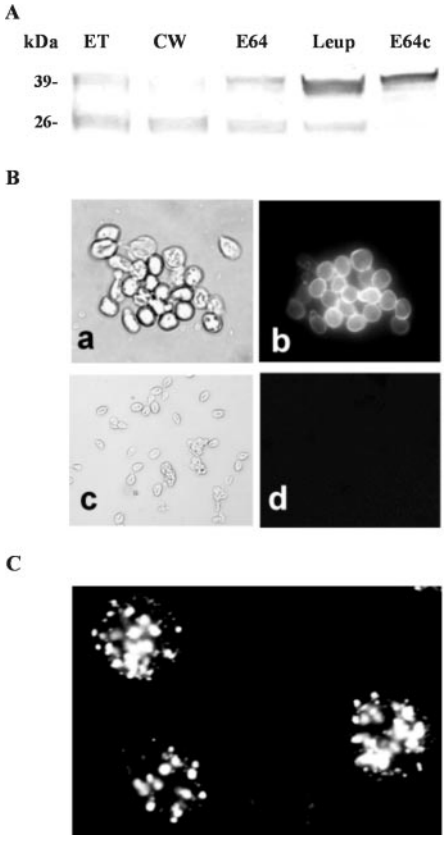

FIG. 1. **Only membrane permeant cysteine proteinase inhibitors block proteolytic processing of CWP2.** *A*, immunodetection of CWP2 by Western blotting using mAb 7D2 on trophozoites after 24 h of encystation in the presence of different protease inhibitors (each lane contains 40 μg of total trophozoite proteins). *ET*, encysting trophozoites; *CW*, purified cyst walls; *E64*, encysting trophozoites in the presence of the non-permeant cysteine protease inhibitor E64; *Leup*, encysting trophozoites in the presence of the partially permeant cysteine inhibitor leupeptin; *E64d*, encysting trophozoites in the presence of the permeant cysteine protease inhibitor E64d. *B*, immunofluorescence assay of trophozoites in process of encystation. Light images are shown in *a* and *c*, fluorescence images in *b* and *d*. Cells obtained from the supernatant of encystation medium in absence of E64d (*a* and *b*) or in presence of the drug (*c* and *d*). In presence of the cell permeant cysteine protease inhibitor, cyst production does not take place. *C*, similar to *B*, but cells were permeabilized to detect CWP2 within secretory granules. Cells appear full of ESVs containing CWP2 but they do not release the granule content to form the cyst wall.

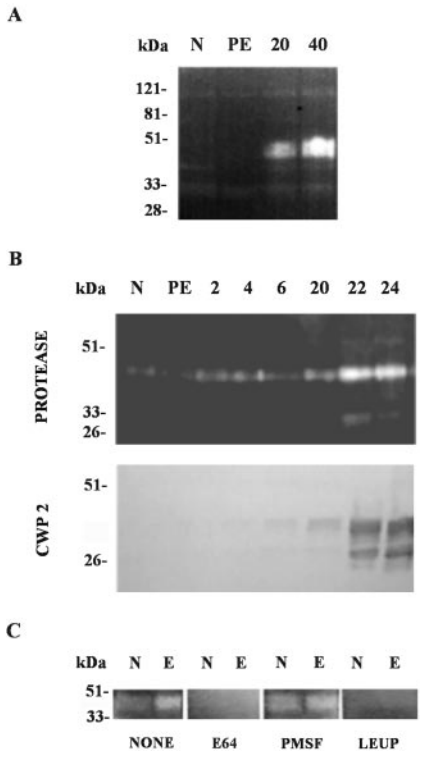

FIG. 2. **Activity of a 45-kDa cysteine protease increases during encystation.** *A*, gelatinolytic activity of cysteine proteases of *G. lamblia* during differentiation into cysts. *N*, trophozoites in growth medium; *PE*, trophozoites in pre-encystation medium; *20* and *24*, trophozoites in encystation medium for 20 and 24 h, respectively. Note that only one proteolytic activity increases during encystation in contrast to several bands which activities remain constant during this process. *B*, comparison of the increase in activity of the 45-kDa cysteine protease during different periods of encystation with the expression of CWP2 by Western blot. *C*, in gel inhibition of protease activity by inhibitors of cysteine proteinases (E64, leupeptin) but not by serine proteinase inhibitors (phenylmethylsulfonyl fluoride). *N,* normal, vegetative trophozoites. *E*, encysting trophozoites.

abolished, in contrast to that observed when phenylmethylsulfonyl fluoride (serine proteinase inhibitor) was used (Fig. 2*C*), indicating that the developmentally induced proteinase was a cysteine proteinase. We thus called this enzyme ESCP (encystation-specific cysteine protease). This enzyme has an optimal pH between 5 and 6 (results not shown).

Purification of the G. lamblia ESCP—To better characterize this enzyme, its purification from 24 h encysting trophozoites was performed using salt precipitations, anion exchange chromatography, and subsequent affinity chromatography using cystatin-Sepharose. The cysteine proteinase was followed by measurements of its molecular weight in polyacrylamide gels stained with silver nitrate and its activity assessed using gelatin-containing gels. Isoelectric point of ESCP was 6.0 as determined by isoelectric focusing before choosing the ion exchange column (Supplementary Material Fig. S1).

Specific Activity of ESCP—The fact that the gelatinolytic activity of ESCP increased during *Giardia* differentiation similar to the increase in CWP2 expression and its correlation with its proteolytic processing suggested that CWP2 could be a substrate of this enzyme. To analyze this hypothesis, recombinant CWP2 (rCWP2) was expressed in *E. coli*, purified, and exposed to encysting trophozoite extracts and the purified ESCP as follows.

First, either rCWP2 or gelatin was co-polymerized with polyacrylamide and the gels used to analyze proteolytic activities of encysting trophozoites. When gelatin was used as substrate, several bands of proteolytic activity were detected, but only one band of ~45 kDa was observed when gelatin was replaced by rCWP2 (Supplementary Material Fig. S2). No band was observed when rCWP1 was used as substrate. Again, among *Giardia* cysteine proteinase activities, that of ~45 kDa, and most likely the one that is developmentally induced during encystation, appears to act on the cleavage of CWP2. Under the conditions described, no other band was observed in rCWP2-indicator gels.

Second, to determine whether the ESCP is specific for CWP2 processing rCWP2 and purified ESCP were confronted in solution under the optimal conditions determined in gels, followed by immunoblotting assays. The action of ESCP on its specific substrate was demonstrated since the 39-kDa form of rCWP2 diminished over time, with the subsequent increase of the 26-kDa polypeptide (Fig. 3).

CWP2 Cleavage Localization—To determine the intracellular organelle in which the proteolytic process takes place, different inhibitors of intracellular protein transport were added to the encystation medium. Then, the percent of cysts produced and the molecular weight of CWP2 were analyzed (Table II). When ammonium chloride, chloroquine (41), brefeldin A (24), or monensin (25) was added, the proteolytic processing of

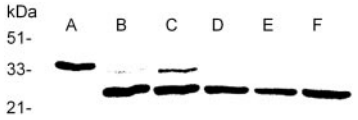

FIG. 3. **Purified ESCP processes CWP2.** In solution cleavage of rCWP2 by purified 45-kDa cysteine protease and subsequent immunodetection by Western blot. Recombinant CWP2 was exposed to 5 μg of purified enzyme in phosphate buffer, pH 6, and incubated at 37 °C for 0 (*A*), 5 (*B*), 15 (*C*), 30 (*D*), and 60 min (*E*). Purified enzyme can cleave the 39-kDa rCWP2 to a 26-kDa fragment in a time dependent manner.

TABLE II
Effect of transport inhibitors on cyst wall formation and CWP2 processing during Giardia encystation

Trophozoites of *G. lamblia* were induced to encyst with or without different vesicular protein transport inhibitors or lysosomotrophic agents. After 24 h, the encystation medium was decanted and the cysts counted and subjected to electrophoresis and Western blotting. Results are as in Table I.

Addition to encystation medium	Cyst production	Molecular weight of CWP2
None	100	39 and 26
Ammonium chloride (500 μM)	0	39
Chloroquine (500 μM)	0	39
Brefeldin A (50 μg/ml)	0	39
Monensin (50 μg/ml)	0	39
Tunicamycin (10 μM)	94	39 and 26
Nocodazole (50 μM)	74	39 and 26
DTT (5000 μM)	0	No expression

CWP2 was inhibited as well as cyst wall formation. Tunicamycin (42) or nocodazole (25) blocked the process partially. The effect of DTT (31) could not be assessed because it was toxic for these cells under the conditions used in this study (Table II).

Association of ESCP with Membranes—The evidence of a possible vesicular localization of the cysteine proteinase prompted us to analyze whether it is a membrane-associated protein. To address this issue, total proteins of trophozoites were analyzed by Triton X-114 partitioning. The 45-kDa band of cysteine proteinase activity corresponding to the ESCP was observed in the detergent fraction (Supplementary Material Fig. S1). This fact and results shown in Table I demonstrating that only permeant inhibitors blocked its activity strongly suggest that ESCP is a cysteine proteinase associated with intracellular membranes.

Molecular Cloning and Sequence Analysis of ESCP—Biochemical and chromatographic techniques allowed the isolation of the 45-kDa ESCP (Supplementary Material Fig. S3), which was then subjected to NH_2-terminal amino acid sequencing. This sequence (DGIGNASQACL) permitted the design of a degenerate oligonucleotide for reverse transcriptase-PCR with RNA isolated from 24 h encysting trophozoites. PCR product was cloned and sequenced. Amino acid sequence deduced from the PCR product nucleotide sequence was highly homologous to several eukaryotic cathepsins. For that reason, the PCR fragment was used to screen a λgt22a *Giardia* cDNA library (10). The longest insert obtained from the library lacked the initiating codon; therefore, 5′-rapid amplification of cDNA ends experiments were performed to obtain the full-length sequence. Results indicated that the transcription initiation starts only two nucleotides upstream the initial ATG. The short 5′-untranslated region is in agreement with most *Giardia* mRNA sequenced so far (43). The full-length sequence was then synthesized by PCR using *Pfu* polymerase (Promega), cloned, and sequenced in both strands. The complete coding sequence for the ESCP cDNA was used to screen a λZAP genomic DNA library (10). Results obtained by subcloning and sequencing the inserts present in two reacting clones confirmed the full-length (1623 bp) ESCP sequence because, like all known *Giardia* genes, it lacks introns (43); in addition, provided additional 5′- and 3′-untranslated sequences (nucleotide sequence data accessible in GenBank™ under the accession number AF293408). A scheme of the predicted protein is shown in Fig. 4. Additionally, Southern blot analysis of genomic DNA probed with the ^{32}P-labeled full-length gene revealed a hybridization pattern consistent with a single copy gene (results not shown). Northern blotting of total RNA allowed the identification of a single mRNA species of about 1600 bp, in agreement with the size of the full-length cDNA, in both encysting and non-encysting trophozoites. Interestingly, the steady-state level of transcripts encoding the ESCP increases during encystation ~20-fold (Fig. 5).

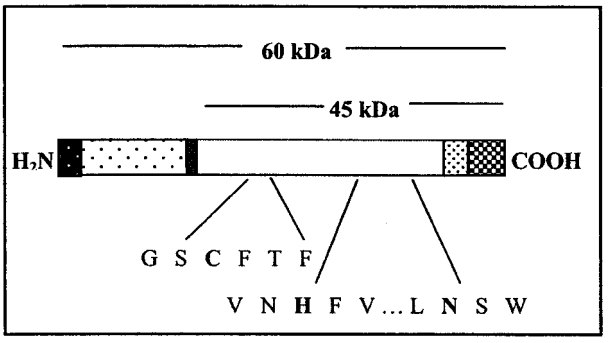

FIG. 4. **Domain structure of the *G. lamblia* ESCP.** Representation of the full-length ESCP based on the predicted amino acid sequence. Amino acids 1–19 of the translated gene correspond to the signal peptide (▩), amino acids 20–110 to the pro-enzyme region (⊡), amino acids 111–122 correspond to the sequenced NH_2-terminal of the mature protein (⊡). The catalytic triad Cys^{268}-His^{433}-Asn^{454} are shown within the catalytic domain (amino acids 241–485; □). The transmembrane domain (▩) and the cytoplasmic tail (▩) are also shown.

The translated nucleotide sequence encoded a 541-amino acid polypeptide with a calculated molecular mass of 60,088 daltons and an estimated isoelectric point for the active enzyme of 5.8, in good agreement with the experimental result obtained by isoelectric focusing (pI 6.0). Computer-assisted analyses predicted a signal peptidase cleavage site between amino acids 19 and 20. Interestingly, this site is 110 residues upstream of the mature amino terminus determined by protein microsequencing. Based on this, we hypothesize that the NH_2 terminus of the ESCP is post-translationally modified by proteolytic processing, and corresponds to the pro-region present in most of the known cathepsins (44). Transmembrane region prediction indicated that the ESCP is an integral protein with a transmembrane domain between amino acids 513 and 529 and a COOH-terminal cytosolic tail from amino acids 530 and 541 (Fig. 4).

BLAST (45) homology search revealed that ESCP shares sequence identity with members of the papain family of cysteine proteinases (peptidase C1), which includes the cathepsin C and B group of cysteine proteinases. Among these, ESCP possesses higher homology with *Schistosoma mansoni*, *Schistosoma japonicum*, rat, human, and mouse cathepsin C-like proteinases (Fig. 6). The amino acid identity of ESCP with homologous from other eukaryotes is in the 28–35% range for the full-length protein and 35–37% range for the peptidase C1 catalytic domain site (amino acids 241–485).

Subcellular Localization of ESCP—Regarding the mechanism involved in vesicle discharge and cyst wall biogenesis, reports have suggested that cyst wall components are released by regulated exocytosis (1, 3), but this proposal have not been accompanied by biochemical and molecular evidences. How ESV release occurs is unknown. To better understand this mechanism, we expressed a tagged version of ESCP in *Giardia* for subsequent immunolocalization of the enzyme during both vegetative growth and encystation. Immunofluorescence results indicated that ESCP is present within vesicles similar to those labeled with the lysosome-specific reagent LysoSensor.

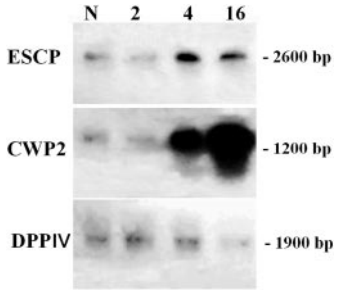

FIG. 5. **Expression of ESCP increases during *Giardia* encystation.** Northern blot analyses of trophozoites cultured in growth medium (*N*) or in encystation medium for 2, 4, and 24 h. The same membrane was blotted with probes for *CWP2* (positive control), Dipeptidyl proteinase IV (constitutively expressed[2]; GenBank™ accession number AF293412); and *ESCP*. Molecular weights are shown at the *right*.

ESCP-containing vesicles were mostly found beneath the cell membrane of both non-encysting trophozoites and those at early stages of encystation, in a pattern consistent with PV localization (Fig. 7). Later during differentiation, however, the enzyme co-localizes with its substrate CWP2 (Fig. 7), suggesting that the PVs interact with the ESVs later during encystation and play an important role during the release before the formation of the cyst wall.

DISCUSSION

G. lamblia exists in two morphologically and metabolically distinct forms: the trophozoite and the cyst (1). In the last decade, many studies have provided important information regarding the mechanisms by which the parasite alternates between these two developmental stages, particularly in aspects related to the stimuli that trigger encystation, the biogenesis of secretory organelles that transport cyst wall constituents, and the identification of some components of the cyst wall (3–5). Nevertheless, little is known about the molecular mechanisms by which cyst wall materials are released and deposited extracellularly (3). For instance, are the ESVs similar to the secretory granules found in higher eukaryotes? Where do they form? Is their content release by direct fusion of the vesicle with the plasma membrane? If it is so, why does the cyst wall of mature cysts rest on top of two membranes, leaving a periplasmic space in between? Where do these membranes come from? How does the dense material of the ESVs form fibrils after discharge? Which factors maintain cyst wall materials without forming fibrils inside the secretory vesicles? Is there any molecule in the granule that impairs fibril formation before ESV release? These are some of the aspects that remain unknown regarding this fascinating mechanism (4).

To get new insights into this process, we first identified and characterized two protein components of the *Giardia* cyst wall, CWP1 and CWP2 (9, 10), and utilized these tools to analyze many aspects of the regulated secretory pathway of this primitive eukaryote (4, 5). In this work, to study the maturation of cyst wall proteins and their association to the biogenesis of the cyst wall, we examined the effect of a series of membrane-permeant and non-permeant proteinase inhibitors during *Giardia* encystation. Our results indicate that a proteolytic event mediated by a cysteine proteinase is involved in processing of CWP2, a required step for cyst wall formation. Since only cell permeant cysteine proteinase inhibitors were effective, this cleavage seems to occur internally, before CWP2 is released from the ESVs.

We also identified, purified, and characterized a cysteine proteinase involved on the processing of CWP2. To demonstrate that the purified enzyme can cleave the authentic precursor we performed *in vitro* experiments using recombinant pro-CWP2 as substrate. Our findings clearly showed that the ESCP is capable of converting CWP2 into its 26-kDa mature form, and that this event is a crucial step in cyst wall formation.

Structurally, ESCP shows characteristics of the cathepsin C family of enzymes. Cysteine and histidine are found in the conserved region of the ESCP corresponding to its active site (46, 47). By contrast, the COOH-terminal region of the molecule differs from other known cathepsins due to the presence of a transmembrane domain and a short cytosolic tail. The catalytic domain of ESCP also possesses high homology to cathepsin B-like enzymes such as those from *Caenorhabditis elegans* (P42507, E: $9 \times e^{-25}$) and *Aztec tobacco* (S60479, E: $4 \times e^{-22}$), among other cysteine proteinases. This supports the fact that although most cathepsin C from higher eukaryotes display exopeptidade activities, ESCP can endoproteolytically process CWP2 due to its homology to cathepsin B-like enzymes, which are endoproteinases. Nevertheless, there are several reports indicating that cathepsin C might also exhibit endoproteolytic activity (48–50).

Interestingly, expression of ESCP is developmentally regulated during encystation of *Giardia*. Proteinase activity increased coincident with the expression of CWP2 and differentiation from trophozoites to cysts. Previously, Yu *et al.* (51) demonstrated that *Giardia* possesses a cysteine proteinase, up-regulated by the viral infection, capable of proteolytic processing of *Giardia* virus capside protein. Although these authors did not further characterize this enzyme, it acts similarly to ESCP. It is likely that *Giardia* virus may cause a cellular response involving the expression of specific genes similar to that occurring in the cell under the encystation stimulus (4, 5).

Proteolytic enzymes have been described in several flagellated protozoan parasites. Cysteine proteinases are always the most profuse protease activities in protozoa and have marked similarity to the major lysosomal cathepsins of vertebrates (52). In *G. lamblia* (53–56), some of these enzymes have been isolated and characterized, showing an optimum pH between 3.5 and 6.0 and ranging in size from 20 to 250 kDa. They also appear to be confined to a particulate fraction in isopycnic centrifugation called peripheral vesicles (PVs) (57, 58). *Giardia* does not have typical lysosomes and PVs seem to fulfill some lysosomal functions (58). Recently, McKerrow's group (57) identified, cloned, and sequenced three cysteine proteinases from *G. lamblia* (all of them of ~30 kDa and homolog to lysosomal cathepsin B) and showed that their inhibition block excystation of *Giardia* cysts *in vitro*. Additionally, fluorogenic substrate labeling indicated that these cysteine proteinases localize to the PVs (57).

Generation of polypeptides from precursor proteins requires not only the coordinated regulation of the expression of the propeptides and the propeptide processing enzymes, but also the coordinate packaging of these proteins into the regulated pathway (46, 47). In encysting *Giardia* trophozoites, the presence of the substrate CWP2 within secretory granules suggests that a CWP2-processing enzyme could be also located in the same organelle. In higher eukaryotes, however, converting enzymes are typically of the serine type, localize into secretory granules, and can be both soluble and membrane associated (59). In contrast, lysosomal proteinases are mostly soluble (43, 46, 47). Since the amino acid sequence predicted by ESCP possesses a transmembrane domain and that in Triton X-114 partitioning its activity is in the hydrophobic fraction, ESCP seems to be membrane-associated, yet being of the cysteine type. In addition, the predicted short cytosolic tail of this proteinase (13-amino acid long) contains the signal YXXϕ, typical of integral membrane proteins that bind adaptor proteins, a requirement for clathrin membrane coating (60), making it difficult to predict where inside the cell CWP2 and ESCP interact.

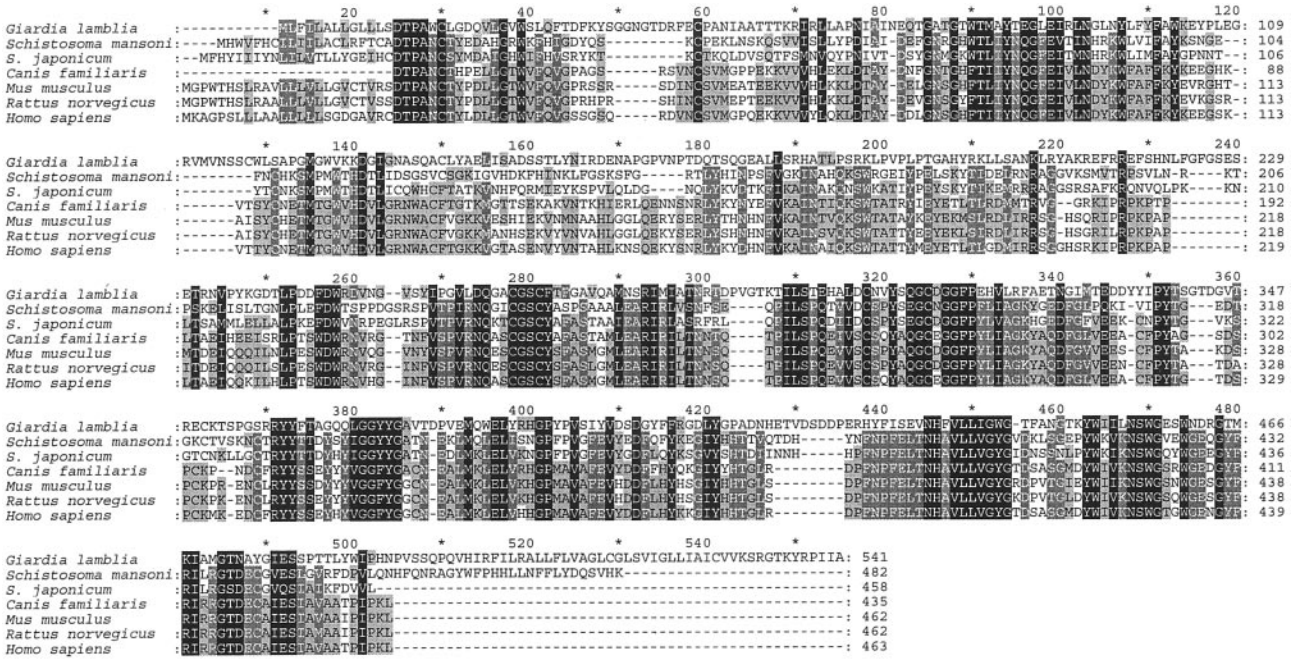

FIG. 6. **Comparison of the deduced amino acid sequence of the catalytic region of ESCP with those of the reported cathepsins of higher eukaryotes.** Alignment was performed using ClustalW 1.8 (72). Cathepsin C from *Schistosoma mansoni* (accession number 2499875), *S. japonicum* (accession number AAC32040.1), *Rattus norvegicus* (accession number 115716), *Mus musculus* (accession number N° AAB58400), *Homo sapiens* (accession number P53634), *Canis familiaris* (accession number AAD02704.1), and the ESCP of *G. lamblia* (accession number AF293408) were included. Labeling was done with the GeneDoc software allowing residue similarity. Percentage of identity among the sequences is represented as shading: *black* (>80%), *dark gray* (79–60%), *light gray* (59–40%), and *no shading* (<39%).

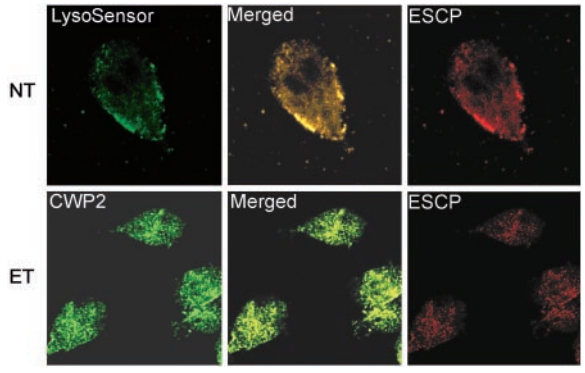

FIG. 7. **Immunolocalization of ESCP in encysting and non-encysting trophozoites.** *Giardia* trophozoites expressing hemagglutinin-tagged ESCP were cultured in either growth (*NT*) or encystation medium (*ET*) for 24 h, then processed for lysosomal labeling using LysoSensor™ (Molecular Probes) and immunofluorescence assays. *Top panel* shows that ESCP localizes into vesicles similar to those labeled with the lysosome-specific reagent LysoSensor™. During cyst wall formation (*bottom panel*), ESCP redistributes within the cells and co-localizes with CWP2 (mAb 7D2), indicating an intimate interaction between these two organelles.

Presence of a signal at the NH_2 terminus of the ESCP indicates that the enzyme is synthesized in the rough endoplasmic reticulum, and after cleavage of the signal peptide, the 541-amino acid precursor is converted into a 58.1-kDa proprotein that is transported along the secretory pathway. The presence of a propeptide similar to other cathepsins C and the lack of activity of this molecule in gelatin-containing gels suggest that activation of the enzyme requires processing of the 110-amino acid propeptide fragment, generating a mature enzyme of 46 kDa. In higher cells, cysteine proteinases of the papain superfamily are synthesized as inactive precursors with 60–110-residue NH_2-terminal prosegments (44, 59). It was suggested that the conversion of pro-cathepsins into their mature forms is carried out in the lysosomes (44, 59) and that the propeptide is a requirement for efficient sorting to these organelles in the absence of mannose 6-phosphate targeting (44, 59–62). It is likely that proteolysis or autoproteolysis of the ESCP induces a conformational change in the inactive precursor and generates an active ESCP *in vivo*. This in fact suggests that a cascade of processing events plays a crucial role in the maturation of CWP2 before it is incorporated into the cyst wall.

PVs of *Giardia* contain lysosomal hydrolases and have been considered as specialized forms of lysosomes in this organism (55, 63–67). The fact that these PVs contain acid phosphatase and can take up ferritin has suggested that they could be involved in endocytosis and food degradation. In a refined structural study, Lanfredi-Rangel *et al.* (64) demonstrated that the PVs of *Giardia* represent both early and late endosomes and lysosomes. It has also been suggested that the PVs might be involved in encystation because their similarity to secretory organelles in other protozoa. Other authors, in contrast, have discounted their role as secretory vesicles in encystation since they noted their abundance after wall formation (65).

Subcellular localization of ESCP was required to understand its transport and the temporal organization of CWP2 processing during encystation. Non-encysting trophozoites expressing hemagglutinin-tagged ESCP display a pattern consistent with PV (lysosomal) localization, like cathepsins from higher eukaryotes (61, 62). In encysting trophozoites, however, some vesicles containing ESCP co-localize with CWP2. Taking into account that only permeant cysteine proteinase inhibitors block CWP2 processing, ESVs might first interact with the PVs and then with the plasma membrane at the time of granule discharge. According to our results, it is obvious that a close interaction between PVs and ESVs must be necessary for the release of secretory granule content before extracellular cyst wall formation. Since immunoelectron localization has shown labeling of PVs with an anti-CWP2 mAb (10), these organelles seem to participate in CWP2 cleavage. Several other studies have shown that trophozoite and cyst proteins such as BiP, protein disulfide isomerase, acid phosphatase, and variant-

specific surface proteins synthesized in the endoplasmic reticulum traffic through the PVs in *Giardia*, indicating that these organelles hold a broad range of physiological functions (3, 9, 10, 53, 65–68).

As reported here, lysosomes seem to play a role in protein secretion through the regulated pathway, at least in primitive eukaryotes. Therefore, the peculiar characteristics of ESCP and CWP2 processing may be important not only as targets for the design of therapeutic agents against this important human pathogen (69–71), but also because subsequent studies on the function and dynamics of these organelles will facilitate a better understanding of the biogenesis and evolution of lysosomes in higher eukaryotes.

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