Sorting of Encystation-specific Cysteine Protease to Lysosome-like Peripheral Vacuoles in *Giardia lamblia* Requires a Conserved Tyrosine-based Motif

María C. Touz‡§, Hugo D. Luñán†, Stanley F. Hayes‡, and Theodore E. Nash‡

From the ‡Laboratory of Parasitic Diseases, NIAID, National Institutes of Health, Bethesda, Maryland 20892, the ¶Cátedra de Bioquímica y Biología Molecular, Facultad de Ciencias Médicas, Universidad Nacional de Cordoba, Cordoba 5000, Argentina, and the §Rocky Mountain Laboratory, NIAID, National Institutes of Health, Hamilton, Montana 59840

Encystation-specific cysteine protease (ESCP) was the first membrane-associated protein described to be part of the lysosome-like peripheral vacuoles in the intestinal parasite *Giardia lamblia*. ESCP is homologous to cathepsin C enzymes of higher eukaryotes, but is distinguished from other lysosomal cysteine proteases because it possesses a transmembrane domain and a short cytoplasmic tail. Tyrosine-based motifs within tails of membrane proteins are known to participate in endosomal/lysosomal protein sorting in higher eukaryotes. In this study, we show that a YRPI motif within the ESCP cytoplasmic tail is necessary and sufficient to mediate ESCP sorting to peripheral vacuoles in *Giardia*. Deletion and point mutation analysis demonstrated that the tyrosine residue is critical for ESCP sorting, whereas amino acids located at the Y+1 (Arg), Y+2 (Pro), and Y+3 (Ile) positions show minimal effect. Loss of the motif resulted in surface localization, whereas addition of the motif to a variant-specific surface protein resulted in lysosomal localization. Although *Giardia* trophozoites lack a morphologically discernible Golgi apparatus, our findings indicate that this parasite directs proteins to the lysosomes using a conserved sorting signal similar to that used by yeast and mammalian cells. Because *Giardia* is one of the earliest branching protist, these results demonstrate that sorting motifs for specific protein traffic developed very early during eukaryotic evolution.

Lysozymes are membrane-bound acidic organelles involved in degradation of endogenous and exogenous macromolecules via biosynthetic or endocytic pathways, respectively (1, 2). In mammalian cells, trafficking between the trans-Golgi network (TGN), endosomes, and lysosomes involves several pathways. Mannose 6-phosphate receptors, TGN-38, furin, sortilin, and other proteins are cycled between the TGN and endosomes without ever reaching the lysosomes. Soluble hydrolases bind to mannose 6-phosphate receptors in the TGN by 6-phosphomannosyl residues and travel to endosomes, where they dissociate from their receptor and subsequently reach lysosomes (3, 4). Structural lysosome-associated membrane proteins are sorted from the TGN to the lysosomes through endosomes by way of tyrosine-based motifs. In addition, other proteins are transported directly to the lysosomes without trafficking through endosomes (5). Yeast, unlike mammalian cells, contains an endosomal or prevacuolar compartment and a large vacuole that functions like a lysosome. Carboxypeptidase Y is transported to the yeast vacuole by its receptor, Vps10p, which returns to the Golgi by the yeast retromer complex. Alternatively, yeast alkaline phosphatase is transported to the vacuole by a different mechanism that avoids the yeast prevacuolar compartment (5).

In yeast and mammalian cells, a clear distinction between early/late endosomes and lysosomes has been established. In contrast, *Giardia lamblia* possesses peripheral vacuoles (PVs) located underneath the plasma membrane that function as endosomes and lysosomes and are therefore considered a primitive endosomal/lysosomal complex (6). These vacuoles, also called peripheral vesicles, are acidic organelles because they can be labeled with lysosomal markers like acridine orange (6) and LysoSensor (7). PVs can take up horseradish peroxidase without delivering it to any other subcellular compartment, suggesting that PVs may be a unique endocytic compartment (6). In addition, numerous soluble enzymes such as acid phosphatase, cathepsins B, and RNase are also present in these vacuoles, indicating their lysosomal nature (8–10); nevertheless, no receptors involved in the sorting of any of these enzymes have yet been described.

Interestingly, PVs are important not only for food degradation, but also for completion of the life cycle of this intestinal parasite. *Giardia* cycles between the disease-causing flagellated trophozoite and the environmentally resistant cyst, which is released with feces and is responsible for transmission of the disease (11). The participation of PVs has also been described to influence secretory granule discharge during cyst wall formation (12), and PVs act as secretary organelles that release cyst wall-disrupting enzymes during excystation (10, 13).

In mammalian cells as well as in yeast, specific sorting signals direct transmembrane proteins to endosomes and/or lysosomes, either from the TGN or from the cell surface, and involve tyrosine-based motifs (YXXΦ, where Φ is any amino acid and Φ is an amino acid with a bulky hydrophobic side chain) and/or acidic cluster dileucine motifs located in their cytoplasmic tails. These motifs can be found in single or multiple copies and also in combination (14). The interaction of...
proteins carrying these motifs with adaptor proteins (APs) and GGA (Golgi-localized, gamma-mannos-containing A) ribosoma-binding factor-proteins) seems to be critical for endosomal/lysosomal protein transport (14, 15).

We recently reported that a cysteine protease of the cathepsin C family is implicated in the processing of a cyst wall protein during encystation and that this enzyme localizes to the peripheral vacuoles of non-encysting Giardia trophozoites (7). This encystation-specific cysteine protease (ESCP) possesses a transmembrane domain and a 12-amino acid cytoplasmic tail, unlike cathepsin C enzymes from higher eukaryotes. In the present study, we show that a tyrosine-based sorting signal (YRP1) within the ESCP cytoplasmic tail functions in the sorting of ESCP to lysosome-like peripheral vacuoles in Giardia.

EXPERIMENTAL PROCEDURES

Expression of ESCP in Trophozoites—To constitutively express ESCP along with three influenza hemagglutinin (HA) epitopes on pTubH7HApac carrying the variant-specific surface protein gene vspH7 (16) was modified to introduce the tag just before the TGA stop codon and EcoRI site immediately following the vspH7 ATG start codon. First, one round of PCR was performed using sense oligonucleotide 5'–ggtaacgctattcaatgtggctatgtagggatac–3' and antisense oligonucleotide 5’–tgacgctatattagaacatcatcagttgtaaacgtcac–3' with the MluI site in boldface, the ApaI site in uppercase, resulting in pTubH7HApac. To exchange the escp gene for the vspH7 gene, pTubH7HApac was digested with ApaI and EcoRV to release vspH7. The DNA fragment corresponding to the entire escp coding region was amplified from Giardia genomic DNA (isolate WB/clone 1267) by PCR using sense 5’–cttggGGCCCTtaatattactgtaaatagctagtaaatagctagtaaatagctagtaaatagctagtaaatagc–3' and antisense 5’-taaatagtattcagtaaatagctagtaaatagctagtaaatagctagtaaatagctagtaaatagctagtaaatagc-3' primers with the ApaI site in uppercase, resulting in pTubESCPHApac. The plasmid was restricted with EcoRV and ligated together, thereby eliminating the sequence corresponding to the ESCP transmembrane domain. The plasmid was restricted with MluI and ligated together. Another round of PCR using the same strategy allowed the insertion of an ApaI site within sense 5’–cttggGGCCCTtaatattactgtaaatagctagtaaatagctagtaaatagctagtaaatagctagtaaatagc–3' and antisense 5’-taaatagtattcagtaaatagctagtaaatagctagtaaatagctagtaaatagctagtaaatagc-3' primers with the ApaI site in uppercase, resulting in pTubESCPHApac. The plasmid was restricted with EcoRV and ligated together, thereby eliminating the sequence corresponding to the ESCP cytoplasmic tail. For K/A, a second EcoRI site was introduced at the end of the sequence coding for the ESCP transmembrane domain. The plasmid was restricted with EcoRV and ligated together, thereby eliminating the sequence corresponding to the ESCP cytoplasmic tail. For K/A, two complementary primers were designed to omit bases 1588–1606. For ΔY/A, the same strategy was followed, introducing a deletion of bases 1606–1624 (see Fig. 5A). For the ΔY/A, ΔK, and ΔK/A point mutations, the corresponding amino acids were replaced with alanine residues (see Fig. 4A). ESCP variants were confirmed by sequencing using dye terminator sequencing (Beckman Coulter).

Immunofluorescence Assays—For fixed cells, trophozoites cultured in growth medium were harvested and processed as described previously (23). Primary anti-HA mAb (Sigma) was used to detect ESCP and ESCP variants, and anti-HA mAb or VSPH7-specific mAb 9104 (27) was used to detect VSPH7 and VSPH7 variants. For assays of viable trophozoites, the cells were washed twice with PBS and 0.1% growth medium and incubated with the specific mAb 9104 for VSPH7 surface localization. For CPW2 localization, mAb T2D (30) was directly labeled with Texas Red (Molecular Probes, Inc., Eugene, OR) following the manufacturer’s instructions and used in encysting trophozoites transfectected with pTubESCPHApac. The specimen were examined with a Zeiss Axioplan fluorescence microscope and/or a Leica TCS-NT/SP confocal microscope. Controls included omission of primary antibody and staining of untransfected cells.

RESULTS

ESCP Localizes to Peripheral Vacuoles in Giardia—HA-tagged ESCP was constitutively expressed in WB/1267 trophozoites. With anti-HA mAb, ESCP showed a PV localization pattern and colocalized with LysoTracker (Fig. 1 and Supplemental Fig. 1), a probe for acidic organelles in living cells (25). To verify that the HA tag does not interfere with ESCP sorting, tagged variants of ESCP carrying V5 and FLAG epitopes as well as green fluorescent protein were expressed and localized to the PVs by immunofluorescence assays using specific mAbs (data not shown). In addition, acid phosphatase (GenBank accession number AA97085) and the variant-specific surface protein VSPH7 tagged with HA localized to the PVs and the plasma membrane.

REFERENCES

Fig. 1. ESCP colocalizes with a lysosomal marker inside the peripheral vacuoles of transfectected trophozoites. Upon immunofluorescence, anti-HA mAb detected the tagged enzyme ESCP in vesicles near the plasma membrane (visualized in green), which colocalized (merge visualized in yellow) with the lysosomal probe LysoTracker (visualized in red). Magnification is ×1000.

M. C. Touz and T. E. Nash, unpublished data.

G. I. Devor and E. B. Schaff, unpublished data.
respectively, indicating that the HA tag at the C terminus does not influence protein trafficking (see below).

**ESCP Sorting Requires a Tyrosine-based Motif**—To analyze whether the YRPI motif located in the cytoplasmic tail of ESCP determines its localization to the PVs, we constructed a series of variants of this enzyme by deletions and mutations (see “Experimental Procedures” and Figs. 2A and 4A). The sorting of these variants was examined by immunofluorescence assays using anti-HA mAb. As was previously documented, ESCP localizes to the PVs in *Giardia* (7), but the truncated version ΔK/A showed surface localization indicated by staining of the trophozoite surface and flagella (Fig. 2B and Supplemental Fig. 2). Expression of ΔK/K, which still has the YXXΦ motif, resulted in no change in ESCP localization (Fig. 2B). In contrast, ΔY/A, which lacks the YRPIIA sequence, relocated the enzyme to the plasma membrane (Fig. 2B). Western blot analysis of total protein extracted from transfected trophozoites confirmed the expression of ESCP and its variants. In every case, 65- and 45-kDa bands corresponding to the immature and mature forms of ESCP, respectively, were observed (Fig. 3) (7). These results prompted us to perform a more detailed analysis of the YRPI sorting signal because only the construct lacking this motif failed to localize ESCP to peripheral vacuoles.

Mutation of YRPI (ΔYRPI) to alanine residues resulted in missorting of the protein to the plasma membrane, showing that this motif is essential for ESCP localization (Fig. 4, A and B). Exchanging tyrosine (ΔY) with alanine localized ΔY to the surface, whereas replacement of the residue that follow tyrosine at position +3 (ΔI) had an intermediate effect because the enzyme was detected both at the surface and in the PVs (Fig. 4, A and B). However, when Arg and Pro were replaced, the enzyme remained in the PVs (data not shown). These results indicate that the tyrosine within the cytoplasmic tail is critical for ESCP peripheral vacuole localization.

The ESCP Cytoplasmic Tail Relocates VSPH7 from the Surface to the PVs—VSPH7 is a variant-specific surface protein of *Giardia* clone GS/M-H7 that possesses a single transmembrane domain and a conserved CRGKA cytoplasmic tail and that covers the entire cell surface, including the flagella (26). VSPH7 is not expressed in *Giardia* clone WB/1267, allowing
The detection of VSPH7 at the surface of transfected WB trophozoites using VSPH7-specific mAb G10/4 (16, 27). First, it was important to determine whether VSPH7 does have also a sorting motif for its plasma membrane localization. In this way, expression of VSPH7-HA (VSPH7 with an HA tag at its C terminus), H7 tagged VSPH7 without its cytoplasmic tail, and H7-AA (tagged VSPH7 with the amino acids in its tail changed to alanine residues) showed the same localization profile on the surface of trophozoites compared with expression of native VSPH7, as determined using either anti-HA mAb (Fig. 5, A and B) or mAb G10/4 (data not shown). Thus, these results show that the HA epitope does not affect VSPH7 localization and that the conserved CRGKA cytoplasmic tail is not involved in VSPH7 plasma membrane sorting.

To analyze whether the ESCP cytoplasmic tail can modify VSPH7 sorting, two different chimeras were expressed in WB/1267 trophozoites: VSPH7-HA possessing the transmembrane domain of ESCP (cH7TM) and VSPH7-HA possessing the ESCP cytoplasmic tail instead of its own conserved tail (cH7CT) (Fig. 6A). Expression of cH7TM resulted in no change in localization because the chimera remained in the plasma membrane (Fig. 6B). Immunofluorescence assay using G10/4, a mAb that recognizes the VSPH7 extracellular domain, confirmed the surface localization of cH7TM in viable trophozoites (Supplemental Fig. 3). These results indicate that the 27-amino acid transmembrane domain of VSPH7 does not contain a specific signal for surface localization and that a protein carrying the 24-amino acid ESCP transmembrane domain is transported to the plasma membrane. In contrast, cH7CT was localized to the PVs, the same subcellular localization as ESCP (Fig. 6C). Moreover, in viable cells, mAb G10/4 failed to detect cH7CT at the surface of the trophozoite (Supplemental Fig. 3).

Taken together, these results show that the cytoplasmic tail of ESCP has all the information necessary to direct proteins to Giardia peripheral vesicles. Furthermore, these findings suggest that a long transmembrane domain is essential for VSPs to be transported to the plasma membrane (see “Discussion”).

ESCP also localizes in Encystation-specific Secretory Vesicles (ESVs) during Encystation—ESCP expression and activity increase during encystation and are involved in the processing of one of the proteins forming the cyst wall (CWP2) (7). Although CWP2 and ESCP colocalize in encysting cells, how and where this interaction takes place are unknown (7). Here, we analyzed ESCP/CWP2 interaction during encystation by immunofluorescence and ESCP subcellular localization by immunoelectron microscopy in encysting trophozoites.

Using directly labeled anti-CWP2 mAb and anti-HA mAb for the detection of ESCP, we found that, at the beginning of encystation, ESCP was in the PVs close to the encysting trophozoite plasma membrane, whereas CWP2 was detected in the ESVs (Fig. 7A). In contrast, during cyst wall formation, both colocalized in the developing cyst wall and in the cyst wall in mature cysts (Fig. 7A).

Immunoelectron microscopy showed ESCP in ESVs as well as on the surface and in the PVs of encysting cells (Fig. 7B and Supplemental Fig. 4). The fact that ESCP could be found inside ESVs close to peripheral vacuoles and its localization at the surface suggest that ESVs interact with PVs during ESV discharge and/or at the time of release onto the surface of encysting trophozoites.

**DISCUSSION**

In higher eukaryotes, the endoplasmic reticulum and the Golgi complex play a central role in the correct protein folding...
and transport. Proteins transported to endosomes or lysosomes are generally sorted away from the trafficking pathway taken by secretory proteins and are instead targeted to the endocytic compartments (28). Giardia does have an endomembranous system that differs from that of higher eukaryotes (29). Giardia lacks organelles that resemble early and late endosomes and instead has peripheral vacuoles with hydrolytic activity. The property of these organelles to accumulate macromolecules and, at the same time, the presence of lysosome-like soluble hydrolases suggest that this parasite possesses an endosomal/lysosomal system represented in this single organelle (6). In addition, these vacuoles seem to perform multiple cellular functions because they also act as secretory organelles at certain points of the Giardia life cycle (7, 13). Despite these differences, in a number of ways, protein transport in Giardia resembles that in higher eukaryotes. One example is the constitutive secretion of VSPs (26, 27) and the regulated secretion of CWPs (23, 30, 31). In addition, signal peptides target VSPs and CWPs through the secretory pathway in Giardia (26, 27), and conserved motifs such as the BiP chaperone/endoplasmic reticulum retention motif (KDEL) are present in this parasite (32). The tyrosine-based motif (YRPI) involved in ESCP transport to the PVs in Giardia is another example of similarity of secretory mechanisms to more evolved cells. In higher eukaryotes,
tyrosine-based signal defines a motif that has the consensus YXX\(\phi\) or NPXY (33). \textit{In vitro} analyses have shown that this motif interacts with the \(\mu\)-subunits of almost all APs described so far (14, 15). Despite that each \(\mu\)-subunit has a preference for 1 amino acid at the \(X\) position favoring a nonpolar, an arginine-rich, and an acidic amino acid for AP1, AP2, and AP3, respectively, there is also an overlapping specificity (YIPL) among them (34). Furthermore, AP \(\mu\)-subunits also have predicitions for the \(\phi\) position, preferring leucine and isoleucine over other hydrophobic amino acids (34). In the case of \textit{Giardia}, the YRPI motif within the ESCP cytoplasmic tail appears to be a putative adaptor-binding domain because it has a proline and an isoleucine at the Y+2 and Y+3 positions, respectively. The exchange of YRPI for alanine residues altered the localization of ESCP from the PVs to the plasma membrane. Moreover, recognition by tyrosine plays a major role in ESCP localization because its replacement was sufficient to relocate the enzyme to other cellular organelles. Point mutation of residues Y+1/Y+2 and Y+3 showed that only the isoleucine at position +3 has a moderate effect on ESCP subcellular localization. It is possible that, as was described for other proteins (15), residues Y+2 and Y+3 may help expose the tyrosine residue to the adaptor subunit, rather than being involved in adaptor recognition.

To better understand protein sorting signals in the primitive eukaryote \textit{G. lamblia}, we performed additional experiments using a type I membrane protein, the variant-specific surface protein VSPH7. When VSPH7 (VSP of \textit{Giardia} clone GS/M) is expressed in \textit{Giardia} clone WB/12,67, it shows a surface pattern (16). Like all VSPs described so far (26, 27, 35–38), VSPH7 possesses a 27-amino acid transmembrane domain and a conserved 5-amino acid cytoplasmic tail (CRGKA). When the 24-residues ESCP transmembrane segment replaced the VSPH7 transmembrane domain, the localization of the VSPH7 chimer remained unchanged. In addition, when VSPH7 lacking the cytoplasmic tail was expressed, the protein appeared on the surface, similar to when the CRGKA tail of VSPH7 was exchanged for 5 alanine residues. These results are consistent with the hypothesis that the length of the transmembrane domain is critical for protein localization, supporting the model wherein short transmembrane domains (≤17 residues) direct proteins to the endoplasmic reticulum and \cis-Golgi, and proteins with long transmembrane domains (≥23 residues) direct proteins to the plasma membrane (39–43). Transmembrane proteins in \textit{Giardia} seem to follow the same criteria. Similar to VSPs, other proteins such as dipetidyl peptidase IV (44) and syntaxin-1 (GenBank\textsuperscript{TM}/EBI accession number AF293409),\(^2\) which have long transmembrane domains, also localized to the plasma membrane, whereas syntaxin-2 (accession number AF293410), a protein with a short transmembrane domain, localized at the Golgi of encysting trophozoites.\(^4\) In addition, a recent report showed that when the VSPH7 transmembrane domain and cytoplasmic tail were added to the extracellular domain of a membrane-anchored SAG1 protein of \textit{Toxoplasma gondii}, the protein was localized to the surface, including the flagella of \textit{Giardia} trophozoites (45). This agrees with the idea that the transmembrane domain, but not a motif or special structure inside the VSPH7 extracellular domain, is critical for its localization. Furthermore, we found that the VSPH7 cytoplasmic tail does not contain a trafficking motif, but, because it is highly conserved, may have an additional unknown function.

When the cytoplasmic tail of VSPH7 was substituted for the ESCP counterpart, the localization of VSPH7 changed from the cell surface to the PVs. These findings suggest that the transmembrane domain directs the transport of membrane-associated proteins in \textit{Giardia} unless they have a sorting signal that specifically routes the protein to another organelle.

There are at least two different mechanisms involved in protein trafficking to the PVs in \textit{Giardia}. This study shows that a conserved tyrosine-based motif in the cytoplasmic tail of ESCP is critical for ESCP localization to the PVs. In contrast, soluble PV proteins such as acid phosphatase and cathepsins B do not contain a tyrosine-based motif. It is possible that these soluble lysosomal proteins, similar to those in the mammalian system, require a receptor-mediated sorting process that involves mannose 6-phosphate receptor- and adaptor-like proteins. Although mannose 6-phosphate receptor-like proteins have not been reported in \textit{Giardia}, proteins with some homology to the \(\alpha\)-subunit (\(\alpha\)-adaptin gene, GenBank\textsuperscript{TM}/EBI accession number AF486293) and \(\gamma\)-subunit (\(\gamma\)-adaptin gene, accession number AF486294) as well as the \(\mu\)-subunit (GiMuA, accession number AAL82729; and GiMuB, accession number AAL)

\(^3\) M. C. Touz, M. J. Nores, N. Gottig, and H. D. Luján, unpublished data.
\(^4\) M. C. Touz, H. D. Luján, and T. E. Nash, unpublished data.
AY078978) of putative APs have been identified, supporting this idea.

It is also well known that the Giardia secretory system undergoes radical changes during encystation (23, 30, 46–48). The most remarkable events are the presence of a well-defined Golgi apparatus and the biogenesis of ESVs that transport newly synthesized CWPs to the plasma membrane for release and cyst wall formation. In the present study, during encystation, ESCP was seen inside ESVs and in the plasma membrane in addition to the PVs. Because ESCP is involved in the processing of CWP2 during cyst wall formation, it is possible that PVs fuse with ESVs where interaction between the enzyme ESCP and the substrate CWP2 takes place. After CWP2 processing, both proteins could then be released by exocytosis in a way that involves a calcium-dependent process (49). The findings presented here confirm the previous suggestion that ESVs interact with PVs during the latter stages of encystation (31).

As an early diverging protist, Giardia seems to have a relatively elementary subcellular organization (11). In particular, it has one of the most basic systems for protein transport and degradation (11). However, it also shares many characteristics with higher eukaryotes, as in the case of conserved sorting motifs. Further studies regarding different sorting signals in Giardia and the molecules interacting with them will provide new insight to better understand the evolution of intracellular protein transport and subcellular organization in eukaryotes and also contribute to defining new targets for therapeutic intervention.

Acknowledgments—We thank Drs. Dennis M. Dwyer and Cecilia Arighi for helpful discussion and John T. Conrad and Liudmila Kula-kova for technical suggestions.

REFERENCES

Sorting of Encystation-specific Cysteine Protease to Lysosome-like Peripheral Vacuoles in *Giardia lamblia* Requires a Conserved Tyrosine-based Motif
Mari?a C. Touz, Hugo D. Luján, Stanley F. Hayes and Theodore E. Nash

*J. Biol. Chem. 2003, 278:6420-6426.*
doi: 10.1074/jbc.M208354200 originally published online December 3, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M208354200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2003/02/19/278.8.6420.DC1

This article cites 49 references, 24 of which can be accessed free at
http://www.jbc.org/content/278/8/6420.full.html#ref-list-1