Adaptor Protein Complex 1 Mediates the Transport of Lysosomal Proteins from a Golgi-like Organelle to Peripheral Vacuoles in the Primitive Eukaryote *Giardia lamblia*

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Giardia lamblia is an early branching protist that possesses peripheral vacuoles (PVs) with characteristics of lysosome-like organelles, located underneath the plasma membrane. In more evolved cells, lysosomal protein trafficking is achieved by cargo recognition involving adaptor protein (AP) complexes that recognize specific amino acid sequences (tyrosine and/or dileucine motifs) within the cytoplasmic tail of membrane proteins. Previously, we reported that *Giardia* has a tyrosine-based sorting system, which mediates the targeting of a membrane-associated cysteine protease (encystation-specific cysteine protease, ESCP) to the PVs. Here, we show that *Giardia* AP1 mediates the transport of ESCP and the soluble acid phosphatase (AcPh) to the PVs. By using the yeast two-hybrid assay we found that the ESCP tyrosine-based motif interacts specifically with the medium subunit of AP1 (G μ a). Hemagglutinin-tagged G μ a colocalizes with ESCP and AcPh and coimmunoprecipitates with clathrin, suggesting that protein trafficking toward the PVs is clathrin-adaptin dependent. Targeted disruption of G μ a results in mislocalization of ESCP and AcPh but not of variant-specific surface proteins. Our results suggest that, unlike mammalian cells, only AP1 is involved in anterograde protein trafficking to the PVs in *Giardia*. Moreover, even though *Giardia* trophozoites lack a morphologically discernible Golgi apparatus, the presence of a clathrin-adaptor system suggests that this parasite possess a primitive secretory organelle capable of sorting proteins similar to that of more evolved cells.

INTRODUCTION

In the early branching protist *Giardia lamblia*, the endomembrane system is reduced to the presence of an endoplasmic reticulum (ER) and lysosome-like peripheral vacuoles (PVs). The property of the PVs to accumulate exogenous macromolecules and, at the same time, lysosome-like soluble hydrolases, suggests that this parasite possesses an endosomal/lysosomal system represented in this single organelle (Lanfredi-Rangel *et al.*, 1998). Although there are obvious differences, protein transport in *Giardia* mimics that of higher eukaryotes because proteins can be secreted constitutively (e.g., variant-specific surface proteins; VSPs) or in regulated manner (e.g., cyst wall proteins; CWP2) (Lujan *et al.*, 1995; Mowatt *et al.*, 1995; Nash *et al.*, 1995). However, unlike yeast and mammalian cells, the lysosomal sorting pathway is not well defined in *Giardia*. In more evolved cells, membrane proteins carrying a sorting motif in their cytoplasmic tails are routed to lysosomes (Bonifacino and Traub,

2003), whereas soluble hydrolases are sorted from the *trans*-Golgi network (TGN) to the endosome/lysosome system through a receptor-mediated process (e.g., mannose 6-phosphate receptors) (Ghosh and Kornfeld, 2003). To recruit these proteins, several adaptor proteins recognize tyrosine-based (YXX ϕ) or dileucine-based motifs in the cytoplasmic tail of transmembrane proteins (Ghosh and Kornfeld, 2003; Bonifacino and Traub, 2003) and also bind clathrin inducing the formation of the clathrin-coated vesicles (CCVs) (Ohno *et al.*, 1995, 1996). There are four heterotetrameric adaptor protein (AP) complexes, AP1, AP2, AP3, and AP4, each composed by four subunits: two large chains (one each of $\gamma/\alpha/\delta/\epsilon$ and β 1-4, respectively), one medium-sized chain (μ 1-4), and one small chain (σ 1-4) (Boehm and Bonifacino, 2002). These AP complexes have been described in different subcellular locations where they may function specifically in cargo selection (Boehm and Bonifacino, 2002). It is well known that AP2 mediates endocytosis from the plasma membrane, whereas AP1, AP3, and AP4 participate in protein sorting from the TGN and/or endosomes to lysosomes.

In *Giardia*, we recently found that a transmembrane PV protein (encystation-specific cysteine protease, ESCP) is transported to the PVs through a tyrosine-based motif (Touz *et al.*, 2003). Moreover, soluble hydrolases such as acid phosphatase (AcPh) and cathepsin B also reside within *Giardia* PVs (Ward *et al.*, 1997; Lanfredi-Rangel *et al.*, 1998; Slavin *et al.*, 2002), although no receptor involved in the traffic of these soluble proteins has been identified. Thus, it is possible that *Giardia* possesses a similar mechanism for lysosomal protein trafficking where adaptor proteins may be involved. The

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Abbreviations used: AcPh, acid phosphatase; CCV, clathrin-coated vesicle; CWP2, cyst wall protein 2; ESCP, encystation-specific cysteine protease; GGD, *Giardia* Genome Database; gDPP, *Giardia* dipeptidylpeptidase; PV, peripheral vacuole; TGN, *trans*-Golgi network; VSP, variant-specific surface protein; YXX ϕ , tyrosine-based motif.

vectors: dsRNA- μ a and Neo-dsRNA- μ a vectors contain 1–1000bp- μ a sequence (r- μ a) between opposing tetracycline-inducible *Giardia* ran promoters' and the puromycin or neomycin acetyl transferase resistant genes flanked by glutamate dehydrogenase (gdh) promoters, respectively.

presence of several secretory protein genes in the *Giardia* genome database (McArthur *et al.*, 2000), including a putative clathrin heavy chain, and two large, one medium, and one small subunits of AP1 and AP2 homologous proteins supports this hypothesis. Probably only AP1 and AP2 participate in protein transport to lysosome-like PVs in *Giardia* because no other adaptor complexes, including AP3 and AP4, the monomeric adaptor GGA, Dab2, epsin, or Hrs (Bonifacino and Traub, 2003) are present in the nearly complete *Giardia* genome.

Here, we show that AP1 is involved in the anterograde protein transport to the *Giardia* PVs but not in the constitutive secretion of VSPs. This finding stands in contrast to the multiple adaptor proteins required for delivery to the endosome/lysosome in more evolved organisms.

MATERIALS AND METHODS

Giardia lamblia Cultivation and Transfection

Trophozoites of the isolate WB, clone 1267 (Nash *et al.*, 1988), were cultured as described previously (Keister, 1983). Encystation of trophozoite monolayer was accomplished by the method described by Boucher and Gillin (1990). Trophozoites were transfected with the constructs by electroporation and selected with puromycin as described previously (Yee and Nash, 1995; Singer *et al.*, 1998; Elmendorf *et al.*, 2001). Cotransfection selection was accomplished by adding 100 μ g of puromycin and 50 μ g of geneticin (Invitrogen, Carlsbad, CA) to the growth medium. For induction of μ a double-stranded RNA (μ a dsRNA), cells transfected with dsRNA- μ a or Neo-dsRNA- μ a vectors were cultured in growth medium with 10 μ g of tetracycline (Sigma-Aldrich, St. Louis, MO) for 24 h. To test the role of *G* μ a during encystation, cells transfected with dsRNA- μ a were encysted for 48 h in the presence or absence of 10 μ g of tetracycline.

Immunofluorescence Assays

For fixed cells, trophozoites cultured in growth medium were harvested and processed as described previously (Lujan *et al.*, 1995). For direct double staining, anti-hemagglutinin (HA) monoclonal antibody (mAb) (Sigma-Aldrich) was conjugated with Zenon One fluorescein and used to detect *G* μ a (final dilution of anti-HA 1:500). Anti-V5 mAb (Sigma-Aldrich), used for ESCP and AcPh detection, was conjugated with Zenon One Texas Red-X (final dilution of anti-V5 1:200) as suggested by the manufacturer (Molecular Probes, Eugene, OR). For VSP9B10, 9B10 mAb (1:200) was used in indirect assays. For cyst detection, the supernatant of 48-h-encysting-cells was washed twice with phosphate-buffered saline (PBS)-0.1% growth medium and incubated with the anti-CWP2-specific 7D2 mAb conjugated with Texas Red as described previously (Touz *et al.*, 2003). Controls included omission of primary antibody and staining of nontransfected cells. The specimens were examined with an Axioplan fluorescence microscope (Carl Zeiss, Thornwood, NY) by using a 40 $\times$ /0.75 (44 03 50) or a 100 $\times$ /1.3 (44 03 50) Plan-NEOFLUAR lens (Carl Zeiss) or Leica TCS-NT/SP confocal microscope. Digital images were recorded using a Hamamatsu digital camera (Hamamatsu, Bridgewater, NJ) and processed with QED Camera Plug-in α (QED Imaging, Pittsburgh, PA).

Figure 1. Schematic representation of *Giardia* vectors. *pTub* μ a-HApac contains the ORF of μ a with the HA epitope sequence at the C terminus. *pTubESCP-V5/6* $\times$ *Hpac* has the ESCP gene and the V5 epitope plus the sequence encoding six His at the C terminus. *pTubAcPh-V5/6* $\times$ *Hpac* carries the AcPh gene and the sequences encoding the V5 epitope plus six His at the C terminus. Vectors carrying two genes (tg): *tgESCP-μa* contains ESCP-V5/6 $\times$ His and μ a-HA; *tgAcPh-μa* contains AcPh-V5/6 $\times$ His and μ a-HA; *tgESCP-CWP2* contains ESCP-V5/6 $\times$ His and CWP2-HA; *tgESCP-gDPP* contains ESCP-V5/6 $\times$ His and gDPP-HA. Each vector possesses a puromycin acetyl transferase gene for selection. All genes are expressed under the control of the tubulin promoter. dsRNA

Immunoprecipitation

HA-tagged *G* μ a transfected *Giardia* trophozoites were disrupted in lysis buffer (50 mM Tris, pH 8.0, 120 mM NaCl, 5 mM EDTA, 1% Triton X-100, and protease inhibitors) for 30 min on ice and centrifuged at 13,000 $\times$ g for 5 min at 4°C. The cell lysate was precleared by using protein A/G-Sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at 4°C, and then subsequently subjected to immunoprecipitation by using 2 μ l of specific anti-clathrin (kindly provided by Dr. Adrian B. Hehl, Institute of Parasitology, University of Zurich, Zurich, Switzerland) (Marti *et al.*, 2003) or anti-aldehyde (our unpublished data) mouse polyclonal antibodies. After incubation overnight at 4°C, protein A/G-Sepharose was added, and the incubation continued for 4 h. The immunoprecipitates were washed three times in lysis buffer before immunoblot analysis.

Immunoblot Analysis

Western blot assays were performed as previously reported (Lujan *et al.*, 1995). Briefly, 10 μ g of total protein/lane from transfected encysting trophozoites were resuspended in 30 μ l of sample buffer (Bio-Rad, Hercules, CA) with 2-mercaptoethanol, boiled for 5 min, and electrophoresed into a 4–12% Tris-glycine polyacrylamide gel. The proteins were transblotted onto polyvinylidene difluoride membranes (Invitrogen) and probed with anti-CWP2 mAb. For pull-down assays, anti-V5 mAb and anti-HA mAb were used at 1:1000 dilution (Sigma-Aldrich). For Immunoprecipitation experiments, anti-HA or anti-VSP9B10 mAbs directed labeled with biotin (Santa Cruz Biotechnology) was used at 1:500 dilution and developed with alkaline phosphatase-conjugated streptavidin (Bio-Rad).

Yeast Two-Hybrid Screening

Construction and screening of a two-hybrid library was done by using the MATCHMAKER library construction and screening kit following the protocol suggested by the company (BD Biosciences Clontech, Palo Alto, CA). Briefly, the two-hybrid GAL4 DNA binding domain (GAL4bd) and GAL4 transcription activation domain (GAL4ad) fusion constructs were prepared by ligating ESCP cDNA or ds cDNA (obtained from total RNA of nonencysting trophozoites; GenBank accession no. AF293408) into the pGBKT7(TRP1) and pGADT7-Rec(LEU2) vectors, respectively. AH109 yeast reporter was cotransformed with pGBKT7, pGADT7-Rec, and ds cDNA by the lithium acetate procedure. For colony growth assays, AH109 transformants were resuspended in water to 0.1 OD 600/ml, and then 5 μ l was spotted on plates lacking leucine and tryptophan (–L/–T) in the presence or absence of histidine (triple dropout medium, TDO) or histidine and adenine (quadruple dropout medium, QDO), and cultured at 30°C for 4–5 d. Sequencing of cDNA inserts was done using dye terminator cycle sequencing (Beckman Coulter, Fullerton, CA). For the yeast two-hybrid assays, the same strategy was used except that ds cDNA of μ a, μ b, *cwp2*, or *gdp* (GenBank accession no. AACB01000112, AACB000044, U28965, and AF293412, respectively) were cloned into pGADT7(LEU2) (BD Biosciences Clontech). Amino acid substitution of ESCP-YRPI motif was made with the QuikChange mutagenesis kit (Stratagene, La Jolla, CA).

Two-Gene Plasmid Construction

To express two tagged *Giardia* proteins at the same time (one carrying the V5/6 $\times$ H and the other the HA epitope at the C termini), we constructed the two-gene plasmid (tg). First, the full-length μ a cDNA encoding *G* μ a protein (nucleotide 1–1345) was cloned in the restricted pTubH7-HApac vector (Touz *et al.*, 2003), yielding pTub μ a-HApac (Figure 1). This vector was then modified by introducing a *SpeI* restriction site before the 5'-tubulin promoter by using a QuikChange mutagenesis kit (Stratagene). To construct the two-gene

plasmid, *escp* (encoding encystation-specific cysteine-protease) or *acph* (encoding acid phosphatase, GenBank accession no. AF293415), including their 5'-tubulin and 3'-tubulin untranslated region controlling sequences, were amplified by polymerase chain reaction (PCR) from pTubESC-V5/6 × Hpac and pTubAcPh-V5/6 × Hpac vectors, respectively (Figure 1), and cloned into the pTub μ a-HA-pac vector by restriction and ligation into the *SpeI* and *XbaI* sites. Thus, the final constructs were tgESC-V μ a and tgAcPh-V μ a that contain V5-tagged ESCP plus HA-tagged Gi μ a and V5-tagged AcPh plus HA-tagged Gi μ a, respectively. The same procedure was followed to create tgESC-CWP2 and tgESC-gDPP for V5-tagged ESCP plus HA-tagged CWP2 or HA-tagged gDPP, respectively (Figure 1). Primers used in the construction of the two-gene plasmid are listed below.

Pull-Down Assays

Giardia transfected with pTubESC-V5/6 × Hpac, tgESC-V μ a, tgESC-CWP2, or tgESC-gDPP (Figure 1) were grown in growth medium (or encysting medium for ESCP-CWP2 protein-protein interaction positive control), harvested, and resuspended in 1 ml of lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazol, pH 8.0, 1% Triton X-100, and protease inhibitors) for 1 h at 4°C. After the lysate was centrifuged at 10,000 × g, 10 min at 4°C, the supernatant was mixed with 200 μ l of Ni-agarose beads (QIAGEN, Valencia, CA) and incubated overnight at 4°C. Beads were spun down at 700 × g and washed four times with wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0, 0.1% Triton X-100, and protease inhibitors). Bound proteins were eluted four times with 100 μ l of elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazol, pH 8.0, 0.1% Triton X-100, and protease inhibitors) and analyzed by immunoblotting.

Giardia dsRNA Vector Construction

A dsRNA vector (our unpublished data) was constructed based on a tetracycline-inducible plasmid made by Sun and Tai (2000) later modified by Elmen-dorf *et al.* (personal communication) exchanging puromycin acetyl transferase for neomycin acetyl transferase gene (Singer *et al.*, 1998). To silence the μ a gene, nucleotides 1–1000 of μ a open reading frame (ORF) (1–1345 nucleotides) were introduced into dsRNA vector by using dsRNA μ aF and dsRNA μ aR primers for μ a amplification (see below). In this plasmid, the 1000-base pair- μ a sequence was introduced between opposing tetracycline-inducible *Giardia* ran promoters, yielding the vector dsRNA- μ a (Figure 1). To cotransform trophozoites and be able to select for each vector, the neomycin acetyl transferase was exchanged for puromycin acetyl transferase gene present in dsRNA- μ a by using the QuikChange mutagenesis kit (Figure 1) (Stratagene).

Slot-Blot Assay

Two micrograms of total RNA extracted from growing trophozoites were immobilized onto a Nytran SuperCharge nylon membrane (Schleicher & Schuell, Keene, NH) by using the MINIFOLD II slot-blot system, according to the manufacturer's instructions. Membranes were first hybridized with antisense probes specific for the last 350 base pairs of μ a gene and then stripped and reprobed by using an antisense specific for tubulin as control. The density of each band was measured using image analysis software EagleSight (Stratagene).

Brefeldin A (BFA) Treatment

To investigate the effect of BFA (Molecular Probes; stock solution 10 mg/ml dimethyl sulfoxide) in the transport of ESCP and Gi μ a, trophozoites were allowed to attach to the slides for 30 min in PBS-0.1% growth medium at 37°C. The PBS-0.1% growth medium was then discarded and fresh solution containing 50 μ g/ml BFA (or dimethyl sulfoxide alone) was added for 1 h at 37°C. After fixation with 4% formaldehyde, the location of ESCP and Gi μ a was determined by immunofluorescence assays as described above.

Statistics

Descriptive statistics included calculation of mean and SD of control (–Tet) and experimental (+Tet) groups. A comparison of the means was performed using independent-samples Student's *t* test. A *p* ≤ 0.05 was considered significant.

Oligonucleotide Primers Used (5'-3' Orientation)

For yeast-two hybrid assay, the following primers were used: ESCP-*XmaI*-A T T C C C G G G T C T T G G G G A C C A A G T T C T G G G C G T A T G G, ESCP-*SalI*-G G G A C A A A A T A C C G T C C A A T A A T T G C A C G T C G A C C T G, ESCP-(YRP1)s-T G C G T A G T T A G T C C C G C G G G A C A A A A g c c g c t g c a g c a A T T G C A C G G A T C C G T C G A C C T G C A G C G, ESCP-(YRP1)as-C G C T G C A G G T C G A C G G A T C C G T G C A A T t g c t g c a g c g g c T T T T G T C C C G C G G G A C T T A A C T A C G C A, μ a-*NcoI*-G T T A C C A T G G T A A G C T C T C T G C T A A T C A T T, μ a-*EcoRI*-G T T G T C A T A A A A C A C A C C A T C G T G G A A T T C C A G, μ b-*NdeI*-G T T T C A T A T G A T C A A G G C G G T C A T T C T T T T G, μ b-*BamHI*-T G C G C G G C C T G T A A C C C A A T C T T G G A

T C C A T C, CWP2-*NdeI*-G T T C A T A T G T G C C C T G C C A C C G A G G A G G A G G C C, CWP2-*BamHI*-A C A A G C C T A T T G T C C G C A G A A G G G G A T C C A T C, DPP-*NdeI*-G T T C A T A T G A T G A C G C T A T C G G C C T G G A T T A T A, and DPP-*BamHI*-T T G A C T G G C T A G A T A C T T A C C T T G G A T C C A T C.

For two-gene vector construction, the following primers were used: μ a-*NcoI*-G A C T C C A T G G T A A G C T C T C T G C T A A T C A T T C A C G G G, μ a-*SfiI*-A T G G T T G T C A T A A A A C A C A C C A T C G T G G G C G C C T A C G, CWP2-*NcoI*-G A C T C C A T G G T C G C A G C C C T T G T T C T A G G A C T T C T T, CWP2-*SfiI*-A C A A G C C T A T T G T C C G C A G A A G G G G C G C C T A C G, DPP-*NcoI*-G A C T C C A T G G C G C T A T C G G C C T G G A T T A T A, DPP-*SfiI*-T T T G A C T G G C T A G A T A C T T A C C T T G G C G C C T A C G, Tub5'-*SpeI*-G T T A C T A G T A T G C A A T G A C G C A C C G C C A A A G A G A C, and Tub3'-*XbaI*-T G C T T G T T C T C T G G C C T G A A A T G G T T A T C T A G A T A C G.

For dsRNA production, the following primers were used: dsRNA μ aF-(*BamHI*)-C A C G G A T C C A T G A A A G C T C T C T G C T A A T C A T T C A C, and dsRNA μ aR-(*SphI*)-T A T T G C C C T C A T G A C A A G T T G C A A G G C A T G C T G G.

Restriction sites are denoted in bold or italics.

RESULTS

Lysosomal-Membrane Protein ESCP Interacts with the Medium Subunit of *Giardia* Adaptor Protein 1 (Gi μ a)

The yeast two-hybrid method was used to screen a *Giardia* cDNA library to detect putative proteins involved in the transport of ESCP to the PVs. The bait strain expressing pGBKT7-ESCP (TRP1) was cotransfected with a nonencysting *Giardia* trophozoite cDNA library in which the inserts were expressed as fusion proteins with the pGADT7-Rec(LEU2) transcription activation domain (see MATERIALS AND METHODS). The initial screen produced 27 positive clones that grew in the selective medium TDO, and 24 positive clones that grew in the selective medium QDO. PCR and restriction digest analysis revealed 10 unique groups among these 24 clones. BLAST search and sequence analysis identified a number of proteins involved in different pathways, including three sequences encoding to a dynein-like protein (GenBank accession no. EAA37885), three encoding a Rab6-interacting protein (GenBank accession no. EAA42818), and, most significantly, five sequences corresponding to the ORF of the medium subunit of *Giardia* adaptor protein 1 (GenBank accession no. EAA38136), called Gi μ a (Marti *et al.*, 2003). These results suggested that the polymeric adaptor protein AP1 is involved in binding and transport of ESCP to PVs in *Giardia*.

Gi μ a Localizes around *Giardia* Nuclei and Colocalizes with ESCP and AcPh in Lysosome-like Peripheral Vacuoles

In mammalian cells, lysosomal-protein transport from the TGN to the endosome/lysosome system is accomplished by inclusion of these proteins into clathrin-coated vesicles in an adaptin-mediated manner (Bonifacino and Traub, 2003). Immunofluorescence assays of constitutively low-expressed HA-tagged Gi μ a (Figure 1) showed this protein surrounding the nuclei and also close to the plasma membrane (Figure 2A). To address the question of whether HA-tagged Gi μ a is integrated into the AP1 complex, specific polyclonal antibodies (pAbs) were added to protein extracts obtained from *Giardia* trophozoites expressing Gi μ a. The results showed that HA-tagged Gi μ a but not VSP9B10 coimmunoprecipitates with the coatome clathrin isolated using anti-clathrin heavy chain pAb. On the other hand, neither HA-tagged Gi μ a nor VSP9B10 was nonspecifically coimmunoprecipitated when an unrelated anti-aldolase pAb was added (Figure 2B).

Direct double immunostaining of trophozoites transfected with two-gene plasmids expressing HA-tagged Gi μ a and V5-tagged ESCP or AcPh (Figure 1) revealed that AP1 par-

Figure 2. *Giμa* colocalizes with PV-resident proteins. (A) Indirect immunofluorescence assays show the HA-tagged *Giμa* surrounding both nuclei and close to the plasma membrane by confocal microscopy. (B) Analyses of immunoprecipitates of *Giardia* lysates formed using *Giardia* polyclonal antibodies to clathrin and aldolase in HA-tagged *Giμa*-expressing organisms. Only immunoprecipitates formed using anti-heavy chain clathrin specifically associates with HA-tagged *Giμa*. (C) Confocal microscopy shows colocalization of HA-tagged *Giμa* (green) and V5-tagged ESCP (red) in the PVs (yellow, merge). Double staining of HA-tagged *Giμa* (green) and V5-tagged AcPh (red) show colocalization of these two proteins around the nuclei and in the PVs in yellow (merge). Magnification, 1000 $\times$.

tially colocalizes with both PV-resident proteins (Figure 2C). The colocalization of AP1 with ESCP and AcPh suggests that *Giμa* is involved in the recruitment from a sorting region that remains undefined (Golgi-like organelle?) and the transport of cargo proteins to the PVs.

ESCP Interacts Specifically with *Giμa* through the YRPI Tyrosine-based Motif

In higher eukaryotes, AP complexes bind to the tyrosine-based or dileucine-based motifs of membrane-associated proteins (Bonifacino and Traub, 2003). Previously, we showed that deletion or mutation of the ESCP tyrosine-based motif (YRPI) causes missorting of ESCP from the PVs to the surface of the trophozoite (Touz *et al.*, 2003). Here, we used the two-hybrid assay to determine whether the YRPI sequence is responsible for the interaction with *Giμa* and whether this motif interacts with the medium subunit of adaptor protein 2 (*Giμb*). Thus, the *μa*, *μb*, *cwp2*, or *gdpp* genes were cloned into pGADT7(LEU2) and full-length *escp* or *escp(-yrpi)* (ESCP where the YRPI sorting motif is replaced by alanines) were cloned into the two-hybrid bait pGBKT7(TRP1). Consistent with our previous findings (Touz *et al.*, 2003), *Giμa* interacts with the full-length ESCP through its tyrosine-based motif (Figure 3, A and B). In contrast, *Giμb* did not bind ESCP (Figure 3, A and B), suggesting that AP1 but not AP2 is involved in transport of

Figure 3. The μ subunit of AP1 interacts specifically with ESCP tyrosine-based motif. (A) Yeast two-hybrid assay shows that the μ subunit of AP1 (*Giμa*) as well as CWP2 (positive control) is able to interact with ESCP, allowing the yeast to grow in selective medium QDO. In contrast, neither the μ subunit of AP2 (*Giμb*) or gDPP (negative control) interacts with ESCP restricting yeast growth to the nonselective medium lacking LEU and TRP (-L/-T). (B) When the YRPI sorting motif of ESCP is mutated to alanines (ESCP-YRPI), *Giμa* is unable to bind to ESCP, showing that the *Giμa*-ESCP interaction depends on YRPI tyrosine-based motif. CWP2 still interacts with the mutated ESCP, indicating that this interaction is independent of the tyrosine-based motif. (C) V5/6 $\times$ His-tagged ESCP was purified using Ni-agarose beads and detected by immunoblotting by using anti-V5 mAb (arrowhead). The presence of associated proteins was analyzed by immunoblotting by using anti-HA mAb. Only *Giμa* and CWP2 were pulled down by ESCP (arrows).

ESCP to the PVs of *Giardia*. The cyst wall protein CWP2 (Lujan *et al.*, 1995) and the surface *Giardia* dipeptidylpeptidase gDPP (Touz *et al.*, 2002c) were used as positive and negative controls, respectively; confirming that ESCP interacts specifically with its substrate CWP2 but in a manner independent of the YRPI motif (Figure 3, A and B) (Touz *et al.*, 2003).

To analyze the interaction between *Giμa* and ESCP, we transfected *Giardia* trophozoites with the following vectors: 1) pTubESCP-V5/6 $\times$ Hpac (expressing V5/His-tagged ESCP alone), 2) tgESCP- μa (expressing V5/His-tagged ESCP plus HA-tagged $\mu 1$ -AP1), 3) tgESCP-CWP2 (expressing V5/His-tagged ESCP plus HA-tagged CWP2), and 4) tgESCP-gDPP (expressing V5/His-tagged ESCP plus HA-tagged gDPP) (Figure 1), and were later subjected to protein pull-down assays. Affinity purified His-tagged ESCP and associated proteins were initially analyzed by immunoblotting with anti-V5 mAb to detect the presence of ESCP (Figure 3C, arrowhead) or with anti-HA mAb to detect associated proteins (Figure 3B, arrows). The analysis showed that the interaction of ESCP with *Giμa* and its substrate CWP2, is both specific and stable in vivo (Figure 3B, ESCP- μa).

Figure 4. Depletion of *Giμa* alters ESCP and AcPh but not VSP subcellular localization. AP1 expression is reduced after production of μ a dsRNA. (A) Two micrograms of total RNA was used for slot-blot assay by using μ a-antisense or tubulin-antisense probes. WB, total RNA of nontransfected trophozoites; NI, total RNA of noninduced transfected trophozoites; I, total RNA of induced transfected trophozoites. (B) Immunofluorescence assay shows *Giμa* expression in noninduced (–tetracycline) and induced transfected trophozoites (+tetracycline). Magnification, 200 $\times$. (C) Confocal microscopy shows that the normal distribution of ESCP in the PVs (–Tet, arrows) is altered after the production of μ a dsRNA (+Tet, arrowheads). AcPh is located around nuclei and in the PVs (–Tet, arrows) but is retained in the sorting place in *Giμa*-depleted cells (+Tet, arrowheads). VSP9B10 does not change its surface localization after *Giμa* depletion. Magnification, 630 $\times$.

Lower Expression of *Giμa* Changes ESCP and AcPh Subcellular Localization

The role of AP1 in transport was further tested by inhibition of *Giμa* expression by using the dsRNA vectors dsRNA- μ a and Neo-dsRNA- μ a (Figure 1). After selection and induction, semiquantitative reverse transcription-PCR revealed an increase of the μ a-antisense RNA production (our unpublished data) as well as a fivefold decrease in μ a-sense RNA as determined by slot-blot analysis (Figure 4A). The ability of dsRNA- μ a to decrease *Giμa* expression was confirmed by inhibition of constitutively expressed HA-tagged *Giμa* by cotransfecting pTub μ a-HA μ a with the inducible Neo-dsRNA- μ a vector. Selection was performed by adding puromycin for pTub μ a-HA μ a and geneticin for Neo-dsRNA- μ a. After induction of the μ a dsRNA with tetracycline, the expression of *Giμa* was dramatically reduced (Figure 4B). These data indicate that both dsRNA- μ a and Neo-dsRNA- μ a vectors decreased *Giμa* expression.

To determine how depletion of *Giμa* affects the sorting and transport to PVs and the surface of *Giardia*, trophozoites were cotransfected with either pTubESCP-V5/6 $\times$ Hpac (expressing V5-tagged ESCP) or pTubAcPh-V5/6 $\times$ Hpac (expressing V5-tagged AcPh) and the Neo-dsRNA- μ a vector. After growing transfected trophozoites with 10 μ g of tetracycline for 72 h, ESCP and AcPh mostly localized to regions close to the nuclei (Figure 4C, arrowheads). In some cases, ESCP was also localized on the surface and colocalized with VSPs (our unpublished data). In contrast, no change was seen in the surface location of VSP9B10 (Figure 4C). Despite changes in location of ESCP and AcPh, there was no effect in the protein levels of ESCP, AcPh, and VSP9B10 in these cells, as determined by immunoblotting (our unpublished data). These data support the active participation of *Giμa* in the transport of PV-resident proteins.

Giμa Disruption Affects the Last Step of the Encystation Process

To survive outside the host, *Giardia* trophozoites differentiate into infective cysts, which are excreted in the feces (Adam, 2001). After the reception of the stimulus that triggers encystation, *Giardia* undergoes several metabolic and morphological changes comprising the synthesis, processing, and transport of proteins (CWPs) that will be secreted and assembled to form a protective cyst wall (Lujan *et al.*, 1995a; Mowatt *et al.*, 1995).

No effect was observed in the growth of *Giμa*-depleted trophozoites; however, these cells fail to accomplish encystation. When dsRNA- μ a transfected trophozoites were induced with tetracycline for 48 h during encystation, the cyst production was significantly reduced (13.9%) compared with noninduced cells (40.3%) (Table 1 and Figure 5A). Analysis of CWP2 (a cyst wall protein highly induced during encystation) by immunofluorescence showed that this protein is present at high level in both cultures.

These results prompted us to analyze the molecular weight of CWP2 by immunoblotting because inhibition of ESCP is known to block the processing (but not the expression) of CWP2 and therefore the formation of the cyst wall (Touz *et al.*, 2002b, 2003). We expected that during *Giμa* depletion, CWP2 would not be proteolytically processed due to the mislocation of ESCP away from the PVs. Immunoblot analysis of cells going through encystation showed that in noninduced cultures CWP2 is processed to a 26-kDa protein, whereas in induced cultures (*Giμa*-depleted cells) CWP2 is not completely processed, remaining mainly as a 39-kDa protein (Figure 5B). Controls of nontransfected trophozoites in the presence or absence of 10 μ g of tetracycline during 48 h of encystation did not show any differences compared with noninduced dsRNA- μ a transfected trophozoites. Therefore, *Giμa* deficiency decreases cyst production due to a failure in CWP2 processing.

Table 1. Targeted depletion of AP1 medium subunit (μ a) decreases cyst production

<i>Giardia</i> trophozoites	Tetracycline induction	No. attached cells/ml	No. nonattached cells/ml	% Encysting trophozoites	% Cyst production
dsRNA μ a		550.000 $\pm$ 10.500	1.00 $\times$ 10 ⁶ $\pm$ 1.000	50.1 $\pm$ 10.5	40.4 $\pm$ 12.5
(I) dsRNA μ a	10 μ g	400.000 $\pm$ 4.500	1.10 $\times$ 10 ⁶ $\pm$ 1.500	80.2 $\pm$ 11.8	13.9 $\pm$ 7.8

Giardia trophozoites transfected with dsRNA- μ a vector were induced (I) to express μ a dsRNA by the addition of 10 μ g of tetracycline to the pre- and encysting medium. After 48 h, the medium was decanted and attached and nonattached cells (including encysting trophozoites and cyst) were harvested separately and counted. The percentage of encysting cells and cysts were determined by immunofluorescence (Touz *et al.*, 2002a). Results represent the mean of 10 independent experiments. For cyst production Student's *t*-test was performed: *t*(18) = 5.69; *p* < 0.001. The 95% confidence interval of difference is from 16.72 to 36.30.

Brefeldin A Blocks ESCP Transport but Not *G* μ a

BFA has proved to be of great value as an inhibitor of protein trafficking in the endomembrane system of mammalian cells by inhibition of the assembly of the COPI and AP1 complexes onto membranes (Sciaky *et al.*, 1997). To analyze whether this metabolite alters ESCP and *G* μ a transport, 50 μ M BFA was added to trophozoites expressing V5-tagged ESCP and HA-tagged *G* μ a. After BFA treatment, ESCP was found close to the nuclei rather than in the PVs (Figure 6A), whereas *G* μ a localization was unaffected (Figure 6B).

DISCUSSION

The protein-transport pathways to compartments of the endosomal/lysosomal system in mammalian, yeast, and protozoa are clearly related (Nothwehr and Stevens, 1994; Hirst *et al.*, 1999). However, the presence of unusual secretory organelles in protozoa, including lysosome-like organelles, argues that there are unique features in their protein trafficking (Lanfredi-Rangel *et al.*, 1998; Hoppe *et al.*, 2000; Waller and McConville, 2002; Ngo *et al.*, 2003). The early branching protist *G. lamblia* possesses one of the most distinctive secretory systems but on the whole is characteristically eukaryotic. For example, the transport is vesicular in

nature and has both constitutive and regulated mechanisms of transport and signaling motifs that function similarly to those of higher eukaryotes. Nevertheless, unlike mammalian cells, *Giardia* lacks a morphologically discernible Golgi apparatus and contains an endosomal/lysosomal system concentrated in one single organelle (PVs) (Adam, 2001; Lujan and Touz, 2003). In a previous study, we reported that transport of a membrane-associated protease ESCP to the PVs is dependent upon a tyrosine-based motif (YXX $\emptyset$), specifically YRPI, residing in its cytoplasmic tail. Similar motifs conforming to YXX $\emptyset$ operate in the transport of membrane-bound lysosomal proteins in higher eukaryotes and therefore suggested conserved mechanisms between *Giardia* and more evolved cells.

In mammalian cells, the medium subunits μ 1, μ 2, μ 3, and μ 4 of the adaptor complex AP1, AP2, AP3, and AP4, respectively, interact specifically with tyrosine-based sorting signals of receptors and other endosomal/lysosomal membrane proteins (Bonifacino *et al.*, 1996; Boehm and Bonifacino, 2001; Bonifacino and Traub, 2003; Luzio *et al.*, 2003). AP1, AP3, and AP4 participate in sorting from the TGN, whereas AP2 mediates transport to the lysosome by endocytosis. These heterotetrameric adaptor complexes, ex-

Figure 5. Depletion of *G* μ a reduces cyst formation. (A) Immunofluorescence assay using direct labeled anti-CWP2 7D2 mAb. Five hours after initiation of encystation both noninduced (-Tet, trophozoites nonexpressing μ a dsRNA) and induced cultures (+Tet, trophozoites expressing μ a dsRNA) show the presence of CWP2 in the ESVs. At 24 h and 48 h of encystation induced cultures show large decreases in cyst production. (B) Immunoblotting by using anti-CWP2 7D2 mAb shows that in noninduced transfected trophozoites (NI) CWP2 is a 26-kDa protein, whereas in induced trophozoites (I), a nonprocessed 39-kDa protein is additionally detected.

Figure 6. Brefeldin A alters ESCP but not *Giμa* localization. (A) Steady-state localization of V5-tagged ESCP in PVs underneath the plasma membrane of vegetative trophozoites (–BFA). When the cells are treated with BFA for 1 h, ESCP is retained around nuclei (+BFA). (B) HA-tagged *Giμa* localization around the nuclei and in the PVs (–BFA) does not change after addition of BFA (+BFA).

cept for AP4, also take part in the recruitment of clathrin to form CCVs (Kirchhausen, 1999, 2000).

Using a variety of complementary techniques, including yeast two-hybrid assays and pull-down studies, we show that ESCP interacts with the medium subunit of *Giardia* AP1 complex (*Giμa*) through the tyrosine-based motif. In addition, we used a unique *Giardia* vector with a tetracycline-inducible promoter coupled with *Giardia*-RAN promoter that allows the inducible expression of μ a dsRNA resulting in a reduction in *Giμa* expression. Analysis of protein subcellular localization in μ a-depleted cells shows that the PV-resident proteins ESCP and AcPh are misplaced. Conversely, surface localization of VSP9B10 is not affected in μ a-depleted cells. More than one adaptor protein is involved in the anterograde lysosomal protein trafficking in higher eukaryotes, whereas in *Giardia*, AP1 seems to be the only one implicated in the transport of transmembrane (ESCP) and soluble (AcPh) lysosomal enzymes from a Golgi-like organelle to the PVs. There is no evidence that other adaptor proteins are involved. First, AP3 and AP4 homologs as well as the monomeric adaptor GGA, Dab2, epsin, or Hrs (Boehm and Bonifacino, 2001) are not present in the essentially completed *Giardia* Genome Database (GGD, <http://jbpc.mbl.edu/Giardia-HTML>). Furthermore, AP2, an adaptor protein that is present in the GGD, does not seem to play a role in the transport of ESCP because AP2 fails to bind to ESCP in the yeast two-hybrid system and AP2 depletion does not alter ESCP localization (our unpublished data). However, AP2 may play a role in PV-protein transport from the plasma membrane (our unpublished data). AP1 is essential for AcPh transport although the receptor mediating transport, analogous to mannose-6-phosphate receptors that mediate transport of soluble lysosomal enzymes in mammalian cells, has not been identified in the GGD nor has any receptor been experimentally detected.

A polyclonal antibody against *Giardia* clathrin heavy chain was able to coimmunoprecipitate clathrin together with

Giμa, suggesting that clathrin is bound to the AP1 complex through its large subunit (*Giβa*) forming “coated pits” (our unpublished data). The AP1- μ a subunit subsequently recognizes a sorting motif within the cytoplasmic tail of the cargo protein that is packaged into CCVs at the place of sorting. Because clathrin (Marti *et al.*, 2003) and AP1 (this work) are also localized to the PVs, it is possible that this complex remains unaltered until it reaches the PVs. Another possibility is that clathrin and adaptor protein complexes play a role in protein sorting from the PVs to different target organelles, similar to the role of the endosomal clathrin coat in protein sorting toward lysosomes (Sachse *et al.*, 2002).

The importance of AP1 for survival and growth differs depending on the specific organism; it seems to play a more essential role in higher eukaryotes than in lower eukaryotes (Nakai *et al.*, 1993; Yeung *et al.*, 1999; Meyer *et al.*, 2000; Shim and Lee, 2000; Shim *et al.*, 2000; Gokool, 2003; Ngo *et al.*, 2003). Although not critical for *Giardia* trophozoite survival and multiplication, AP1 is necessary for cyst formation. AP1 may act indirectly in this process by transporting ESCP to the PVs, allowing it to cleave the carboxyl tail of the CWP2. This event is critical for mature cyst wall formation (Touz *et al.*, 2002b, 2003). Another less likely possibility is that AP1 is involved in the formation of encystation-specific secretory vesicles (ESVs) because secretory vesicles in many evolved cells contain clathrin-adaptor proteins together with other coat proteins at the time of vesicle formation (Tooze and Tooze, 1986; Molinete *et al.*, 2000; Tooze *et al.*, 2001). We do not favor the second explanation because we observed that ESV formation and CWP2 expression are not affected in AP1-deficient cells. AP1 could also be critical during the process of excystation because the AcPh seems to perform a crucial role during this step of cell differentiation (Slavin *et al.*, 2002). AP1-deficient trophozoites are unable to encyst, implying that disruption of the AP1 function can serve as a potential target to block *Giardia* transmission.

Using the yeast two-hybrid system, we identified a number of proteins in nonencysting trophozoites that interacts with ESCP. Most are homologs of proteins that play a known role in the secretory processes and are associated with Golgi and Golgi functions. The interactions of ESCP and proteins known to be Golgi associated in higher eukaryotes suggest the existence of an organelle-specific membrane complex analogous to the Golgi complex in *Giardia*. Furthermore, cells treated with BFA, which inhibits the assembly of the COPI and AP1 complexes onto membranes in mammalian cells, show a redistribution of ESCP to the ER but not of *Giμa*, suggesting that ESCP is transported to the PVs first through a Golgi-like compartment in COPI-coated vesicles and then assembled into AP1/clathrin-coated vesicles in a post-Golgi compartment resistant to BFA. Our results are consistent with the presence of an organelle developed enough to select proteins to be packaged and delivered to different final destinations in the vegetative form of *Giardia*. Whether this organelle with sorting function is a specialized ER, a primitive Golgi, or TGN organelle or even a subdomain of the nuclear envelope remains to be determined.

In summary, we show that AP1 is specifically involved in the anterograde protein transport to the lysosome-like PVs in *Giardia*. Furthermore, the presence of homologues associated with Golgi that play a role in vesicular transport, the existence of similar activities of inhibitors of Golgi function as well as sorting signals analogous to higher eukaryotes, all suggest the presence of a Golgi or Golgi-like organelle in *Giardia* trophozoites. Because *Giardia* is an early branching protist, the study of vesicular transport in this parasite will

lead to a clearer understanding of the minimal machinery required for protein transport in more evolved cells.

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