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Active and Passive Mechanisms Drive Secretory Granule Biogenesis during Differentiation of the Intestinal Parasite *Giardia lamblia*[*] [S]

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The parasitic protozoan *Giardia lamblia* undergoes important changes to survive outside the intestine of its host by differentiating into infective cysts. During encystation, three cyst wall proteins (CWPs) are specifically expressed and concentrated within encystation-specific secretory vesicles (ESVs). ESVs are electron-dense secretory granules that transport CWPs before exocytosis and extracellular polymerization into a rigid cyst wall. Because secretory granules form at the *trans*-Golgi in higher eukaryotes and because *Giardia* lacks an identifiable Golgi apparatus, the aim of this work was to investigate the molecular basis of secretory granule formation in *Giardia* by examining the role of CWPs in this process. Although CWP1, CWP2, and CWP3 are structurally similar in their 26-kDa leucine-rich overlapping region, CWP2 is distinguished by the presence of a 13-kDa C-terminal basic extension. In non-encysting trophozoites, expression of different CWP chimeras showed that the CWP2 basic extension is necessary for biogenesis of ESVs, which occurs in a compartment derived from the endoplasmic reticulum. Nevertheless, the CWP2 basic extension *per se* is insufficient to trigger ESV formation, indicating that other domains in CWPs are also required. We found that CWP2 is a key regulator of ESV formation by acting as an aggregation factor for CWP1 and CWP3 through interactions mediated by its conserved region. CWP2 also acts as a ligand for sorting via its C-terminal basic extension. These findings show that granule biogenesis requires complex interactions among granule components and membrane receptors.

Giardia lamblia, a parasitic protozoan of humans and other vertebrates, is a major source of waterborne disease worldwide. Clinical signs of giardiasis vary from asymptomatic infection to acute or chronic disease associated with diarrhea and malabsorption. *Giardia* is also of biological interest because it derives from one of the earliest branches of the eukaryotic line of descent (1).

Giardia undergoes important biological changes to survive in hostile environments, alternating between the motile trophozoite and the environmentally resistant cyst (see Fig. 1) (1, 2). Trophozoites inhabit the upper small intestine and are responsible for symptoms of the disease, whereas cysts develop in the lower intestine and are excreted with the feces. This allows *Giardia* survival outside the intestine and transmission among susceptible hosts (1).

The encystation process includes cyst wall component synthesis and secretory organelle biogenesis. Encystation-specific secretory vesicles (ESVs)[2] (3–5), absent in non-encysting trophozoites, are necessary to transport cyst wall secretion components, leading to assembly of the extracellular cyst wall (1).

We previously characterized two *Giardia* cyst wall proteins (CWPs): CWP1 and CWP2 (6, 7). A recent *Giardia* Genome Database search identified a new cyst wall protein, CWP3 (8). CWP1, CWP2, and CWP3 expression increases after trophozoites are exposed to the encystation stimulus (6–8). CWP1, CWP2, and CWP3 are acidic proteins of 26, 39, and 27 kDa, respectively. A hydrophobic N-terminal signal peptide targets them to the secretory pathway (6–9). The central region of CWP1 and CWP2 consists of five tandem leucine-rich repeats, whereas CWP3 has four complete and one incomplete leucine-rich repeat (8). Leucine-rich repeat motifs in both prokaryotic and eukaryotic proteins have diverse functions and cellular localizations, but are always implicated in protein/protein interactions (10). The C terminus of CWPs has a cysteine-rich domain involved in the formation of disulfide-bonded oligomers (7). Although CWPs are closely related to each other, CWP2 is distinguished from CWP1 and CWP3 by the presence of a basic 121-residue C-terminal extension. In CWP2, this C-terminal region is present within ESVs, but is proteolytically cleaved before cyst wall assembly. The C-terminal processing role of CWP2 in early encystation is unknown (2, 3, 7).

After synthesis in the endoplasmic reticulum (ER), CWPs are shuttled to the cell exterior within ESVs. ESVs are large, morphologically irregular, electron-dense granules that form *de novo*, and their presence is the earliest morphological change observed during *Giardia* encystation (Fig. 1) (4, 11, 12). In cells from higher eukaryotes, regulated secretory proteins concentrate into a dense core that buds off, forming an immature secretory granule in the last portion of the Golgi apparatus (13–15). The Golgi apparatus consists of flattened cisternal membranes forming a stack and is remarkably conserved throughout eukaryotic evolution (16); however, a typical Golgi complex is not apparent in vegetative *Giardia* trophozoites (1). Evidence suggests that *Giardia* may possess organelle(s) in which typical Golgi functions take place, even though they do not have a Golgi-like appearance (1). Constitutive and

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[2] The abbreviations used are: ESVs, encystation-specific secretory vesicles; CWPs, cyst wall proteins; ER, endoplasmic reticulum; TGN, *trans*-Golgi network; VSP, variant-specific surface protein; HA, hemagglutinin; TM, transmembrane domain; mAbs, monoclonal antibodies; FITC, fluorescein isothiocyanate; DAPI, 4′,6-diamidino-2-phenylindole.

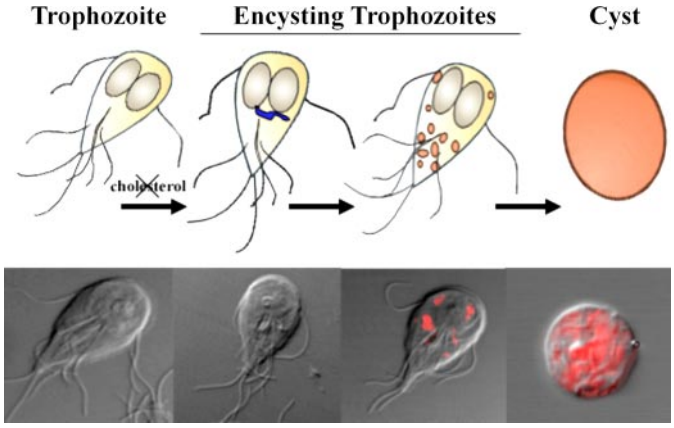

FIGURE 1. ***Giardia* trophozoite differentiation into cysts.** *Upper* and *lower panels*, schematic representation and micrographs, respectively, depicting each step of the encystation process. *Giardia* differentiation is initiated when cells, after a period of culture in a bile-free medium called pre-encystation, are deprived of cholesterol or challenged with high bile concentrations. The earliest morphological change observed in encysting trophozoites is the appearance of large secretory granules called encystation-specific secretory vesicles, which transport cyst wall materials (in *orange*) for subsequent release and extracellular assembly of the rigid cyst wall that protects the parasite outside its host.

regulated mechanisms for protein transport exist in *Giardia*, suggesting Golgi functions, because the sorting and selection processes generally occur in the *trans*-Golgi network (TGN) (17). The *Giardia* constitutive secretory pathway occurs by variant-specific surface protein (VSP) continuous transport to the plasma membrane and extracellular release. Additionally, hydrolytic enzyme sorting to lysosome-like peripheral vesicles is also a component of the constitutive pathway (3, 11, 18). Nonetheless, knowledge of the regulated secretory pathway induced during *Giardia* encystation is limited and controversial (19, 20).

Immunoelectron microscopic studies indicate that synthesized CWPs are concentrated within flattened cisternae. These cisternae increase in size, forming large (>1-μm diameter) membrane-bound ESVs (7, 12, 21). Detailed structural analyses of encysting cells (22) and the presence of BiP, an ER-resident chaperone, in these organelles (23, 24) suggest that ESVs arise from modified ER cisternae. Whether these specific secretory granules form from an uncharacterized *trans*-Golgi or through condensation within the ER is unclear (20, 22).

Previously, we (6, 7) and others (12, 22) observed that CWPs aggregate within membrane-bound clefts. These aggregates appear to grow by direct addition of newly synthesized CWPs, forming large ESVs, suggesting that CWP accumulation is an important factor for granule formation. During encystation, inhibition of CWP synthesis abolishes ESV formation (25). Furthermore, blocking CWP transport at low temperatures indicates that ESV formation depends on CWP export from the ER (26).

We hypothesized that ESV formation is a direct consequence of CWP synthesis (20). We investigated the molecular basis of secretory granule formation by examining the role of CWPs and CWP chimeras in ESV biogenesis in *Giardia*. Our results suggest that secretory granule formation requires complex interactions between granule components (aggregation/condensation) and granule membrane receptors (sorting), involving both passive and active mechanisms.

MATERIALS AND METHODS

Construction of Expression Vectors—For CWP expression in *Giardia*, the corresponding genes were amplified with sense primers containing NcoI or ApaI sites and antisense primers containing EcoRV or SmaI sites. The products were purified, digested, and cloned into the pTubH7HApac vector as reported previously (18).

Strategy and Oligonucleotides Used to Construct Different CWP Variants—To constitutively express *cwp1*, *cwp2*, *cwp3*, and *cwp2* without the basic extension (*cwp2*($-T_{CWP2}$)), all fragments were PCR-amplified from genomic DNA with the following sense (s) and antisense (as) oligonucleotides and cloned into the pTubHApac vector: CWP1s, 5′-CCA CCA TGG TGA TGC TCG CTC TCC TT-3′; CWP1as, 5′-GTT GAT ATC AGG CGG GGT GAG GCA G-3′; CWP2s, 5′-CCA CCA TGG TCG CAG CCC TTG TTC-3′; CWP2as, 5′-AGC GAT ATC CCT TCT GCG GAC AAT AGG-3′; CWP3s, 5′-CCA CCA TGG TTT CTC TGC TTC TTC TCC-3′; CWP3as, GTT GAT ATC TCT GTA GTA GGG CGG CTG-3′; CWP2-Ts, CCA CCA TGG TCG CAG CCC TTG TTC-3′; and CWP2-Tas, 5′-GTT GAT ATC GAC TAC TGT CTG CCT GTA GTA-3′.

To constitutively express the CWP2 basic extension (T_{CWP2}), the signal peptide of CWP2 was added in front of T_{CWP2}. For this purpose, the fragment corresponding to the basic extension was amplified with a sense primer containing an XhoI site (TAILs, 5′-ACT CTC GAG AGA GAT GGA TGC ACG-3′) and antisense primer CWP2as. The fragment corresponding to the signal peptide was amplified with sense primer CWP2s and an antisense oligonucleotide containing an XhoI site (Spas, 5′-ATT CTC GAG AGC GGC GCG AGC A-3′). The two fragments were purified, digested with XhoI, and then ligated. The ligated product was re-amplified using primers CWP2s and CWP2as and cloned into the pTubHApac vector.

The chimera *cwp1*($+T_{CWP2}$)-hemagglutinin (HA) was generated by PCR. *cwp1* was amplified using primer CWP1s and an antisense oligonucleotide with a 5′-region complementary to the beginning of the *cwp2* basic extension (MIX1, 5′-CTT TCG TCC CGA CGC ATT GCG AGG CGG GGT GAG GCA GTA C-3′). The basic tail (T-HA) was amplified using a sense oligonucleotide with a 5′-region complementary to the end of *cwp1* (MIX2, 5′-GTA CTG CCT CAC CCC GCC TCG CA ATG CGT CGG GAC GAA AG-3′) and antisense primer CWP2as. The two fragments *cwp1* (726 bp) and T_{CWP2} (363 bp) were purified and hybridized by a denaturation-hybridization round (95 °C for 2 min, 65 °C for 1 min, and 73 °C for 10 min). The product was re-amplified by PCR using primers CWP1s and CWP2as and cloned into the pTubH7HApac vector.

Strategy and Oligonucleotide Primers Used to Construct Different VSPH7 Chimeras—*vspH7* without its transmembrane domain (*vspH7*($-$TM)-HA) was PCR-amplified from genomic DNA with the following sense (s) and antisense (as) oligonucleotides and then cloned into pTubH7HApac: VSPH7-TMs, 5′-ATC GGG CCC ATG TTT CTA TTA ATT AAT TG-3′; and VSPH7-TMas, 5′-AGC GAT ATC GGA GAG GTT GGG GCC AC-3′. The chimera of *vspH7* to which the basic extension of *cwp2* was added, *vspH7*($-$TM$+T_{CWP2}$)-HA, was generated by PCR using the QuikChange site-directed mutagenesis kit (Stratagene) following the protocol described by Geiser *et al.* (27). Briefly, *vspH7* cloned in pTubH7HApac was modified to construct the chimera using primers that have sequences complementary to *vspH7*, to the basic extension of *cwp2*, and to the vector. The antisense oligonucleotide used had a 5′-region complementary to the vector and a 3′-region complementary to the end of *cwp2* (TAILas, 5′-CAG GCA CAT TCA TAT GGA TAG ATA TCC CTT CTG CGG ACA ATA GGC TTG TTC-3′). The sense oligonucleotide had a 5′-region complementary to the region of interest of *vspH7* and a 3′-region complementary to the beginning of the *cwp2* basic extension (VSPH7-TM+Ts, 5′-CGG CGA TAG TGG CCC CAA CCT CTC CCT CGA GAG ATG GAT GCA CGT AC-3′). All constructs were verified by sequencing.

Giardia Culture and Transfection—Clone WB/1267 trophozoites (28) were cultured and induced to encyst as described previously

(18). Trophozoites were transfected with the constructs by electroporation and selected with puromycin as described (18). After the transfection, clones with low expression were selected by immunoblot and immunofluorescence analyses. For transient coexpression, trophozoites were transfected with both plasmids at the same concentration (10 μg) and selected with puromycin, and clones were analyzed with the corresponding anti-CWP1 and anti-CWP2 monoclonal antibodies (mAbs).

Giardia Trophozoite Immunofluorescence Analysis—Cells cultured in growth, pre-encystation, or encystation medium were harvested and processed as described previously (7). The cells were fixed with 4% paraformaldehyde and permeabilized for 1 h at room temperature in phosphate-buffered saline, 0.1% Triton X-100, and 10% normal goat serum. The cells were then incubated with the antibodies diluted in phosphate-buffered saline, 0.1% Triton X-100, and 3% goat serum. For indirect staining, slides were incubated with the specific mAbs (final dilution of 1:200) or anti-HA mAb (final dilution of 1:1000; Sigma) for 1 h at 37 °C, followed by anti-mouse secondary antibody labeled with fluorescein isothiocyanate (FITC) or rhodamine (final dilution of 1:250; ICN Biomedicals) for 1 h at 37 °C. For direct double staining, FITC-conjugated anti-HA mAb (final dilution of 1:500) was used to detect the transgenic proteins; mAb 9C9 was directly labeled with Texas Red (Zenon One, Molecular Probes) for GRP78/BiP detection; and mAbs 5-3C and 7D2 were directly labeled with Texas Red or FITC (Zenon One) for endogenous CWP1 and CWP2 detection. The nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI). Controls included no primary antibodies and untransfected cells. Confocal images were collected using a Zeiss LSM5 Pascal laser-scanning confocal microscope equipped with an argon/helium/neon laser and a ×100 (numerical aperture = 1.4) oil immersion objective (Zeiss Plan-Apochromat). Single confocal sections of 0.3 μm were taken parallel to the coverslip (*z* sections). Images were acquired using a Zeiss charge-coupled device camera and processed with LSM and Adobe Photoshop software.

Immunoblot and Secretion Assays—SDS-PAGE of total *G. lamblia* proteins was performed under reducing or nonreducing conditions as reported previously (7). For Western blot comparative analysis between different CWPs and CWP chimeras, five times more protein from transgenic trophozoites (100 μg instead of 20 μg) was loaded onto the gel because of the lower level of expression of the constructs expressed by the pTubHApac vector compared with CWP expression during encystation. For secretion, transfected cells were cultured in growth medium for 24 h. The cultured medium was collected and centrifuged at 800 × *g* for 10 min to eliminate *Giardia* trophozoites. The collected medium was incubated with trichloroacetic acid (10% final concentration) for 1 h at 4 °C. Trichloroacetic acid precipitates were centrifuged, and the resulting pellets were washed with iced-cold ethanol, dried, resuspended in 30 μl of sample buffer with 2-mercaptoethanol, and boiled for 5 min. The samples were analyzed by Western blotting using anti-HA mAb.

Electron Microscopy—Cultures were fixed *in situ* to preserve the cell organization when attached to the culture flask wall. After 30 min in fixative (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.0), the flask wall was lightly scraped to detach the trophozoites. The resulting suspension was centrifuged at 500 × *g*, and the pellet was placed in fresh fixative. Fixation was performed for 2 h, followed by post-fixation in 1% OsO_4 with the addition of 1.25% potassium ferrocyanide for 1–2 h. During dehydration, the cells were prestained with 1% uranyl acetate in 70% ethanol for 2 h. The pellets were embedded in Araldite, and sections were cut in a Poter-Blum ultramicrotome. Thin, silver interference color (~60 nm in thickness), serial sections were used. Single whole grids (oval, 2 × 1 mm; Pelco International) were used for collecting segments of 20 sections from series of 100–120 thin sections. The sections were stained first with saturated uranyl acetate in water and then with lead citrate. Sections were examined in a Siemens Elmiskop at magnifications standardized with diffraction grids.

RESULTS

The CWP2 Basic Tail Is Necessary for Secretory Granule Biogenesis—To analyze CWP involvement in ESV biogenesis, different tagged versions of CWPs (Fig. 2*A*) were constitutively expressed in non-encysting trophozoites (Fig. 2*B*). We used a C-terminal HA epitope tag because CWPs have an N-terminal signal peptide that is processed during their trafficking through the secretory pathway (6, 7). After transfection, we analyzed protein expression by Western blotting and immunofluorescence microscopy using either anti-HA or anti-CWP mAb. The expression level of the CWP chimera was always lower than that of endogenous CWP during encystation (~5-fold lower compared with CWP expression in wild-type encysting cells) (data not shown). Localization of HA-tagged CWP during encystation was similar to that of native CWPs (see below), indicating that the HA tag did not affect their subcellular localization. Immunofluorescence assays using anti-HA mAb with constitutively HA-tagged CWP1 and CWP3 showed that both proteins localized to the ER in non-encysting trophozoites (Fig. 2*B*), as determined by their co-localization with the ER-resident chaperone BiP (23). When the same assay was performed with HA-tagged CWP2, formation of large vesicles with characteristics similar to those of native ESVs was observed (Fig. 2*B*). Like ESVs in encysting trophozoites, these vesicles contained BiP (Fig. 2*B*). CWP2 differs from CWP1 and CWP3 by the presence of a 13-kDa basic extension at the C-terminal end (7). We deleted the CWP2 basic tail (T_{CWP2}) to examine its role in ESV biogenesis. HA-tagged CWP2 without the basic extension (CWP2($-T_{CWP2}$)-HA) and CWP1 containing the CWP2 extension at its C terminus (CWP1($+T_{CWP2}$)-HA) were expressed in trophozoites (Fig. 2*A*). Immunofluorescence assays performed on non-encysting cells showed that CWP2 minus the basic extension localized in a cytoplasmic meshwork resembling the ER, a pattern similar to those of CWP1-HA, CWP3-HA, and BiP (Fig. 2*B*). The formation of ESV-like vesicles in cells expressing CWP1($+T_{CWP2}$)-HA was similar to that observed in non-encysting trophozoites with CWP2-HA (Fig. 2*B*) or in encysting trophozoites with wild-type CWP2 (7). These vesicles also co-localized with BiP (Fig. 2*B*). We confirmed the aforementioned localization with additional mAbs that detect CWP conformational states. These data suggest that the exogenous proteins undergo normal folding (supplemental Fig. 1). Negative staining in non-encysting trophozoites with mAb against granule-specific protein, an ESV-specific calcium-binding protein induced during encystation (29), confirmed that these cells were not encysting (data not shown).

In encysting cells, HA-tagged CWP1 and CWP3 constructs were sorted to secretory granules and co-localized with endogenous CWPs (Fig. 3*A*), indicating that native CWP2 reroutes CWP1 and CWP3 from the constitutive to the regulated pathway. In addition, immunofluorescence assays with anti-HA mAb were performed to confirm that these ESV-like granules behave like native ESVs. These experiments showed that CWP1-HA, CWP3-HA, and CWP2($-T_{CWP2}$)-HA were incorporated into the cyst wall similar to native CWPs (Fig. 3*B*). CWP2-HA and CWP1($+T_{CWP2}$)-HA were not detected in cyst walls using anti-HA mAb, in agreement with previous results showing that this basic extension is cleaved from CWP2 by an encystation-specific cysteine protease before cyst wall assembly (30). In addition, the cysts generated *in vitro* from transfected cells were identical in shape to those obtained from

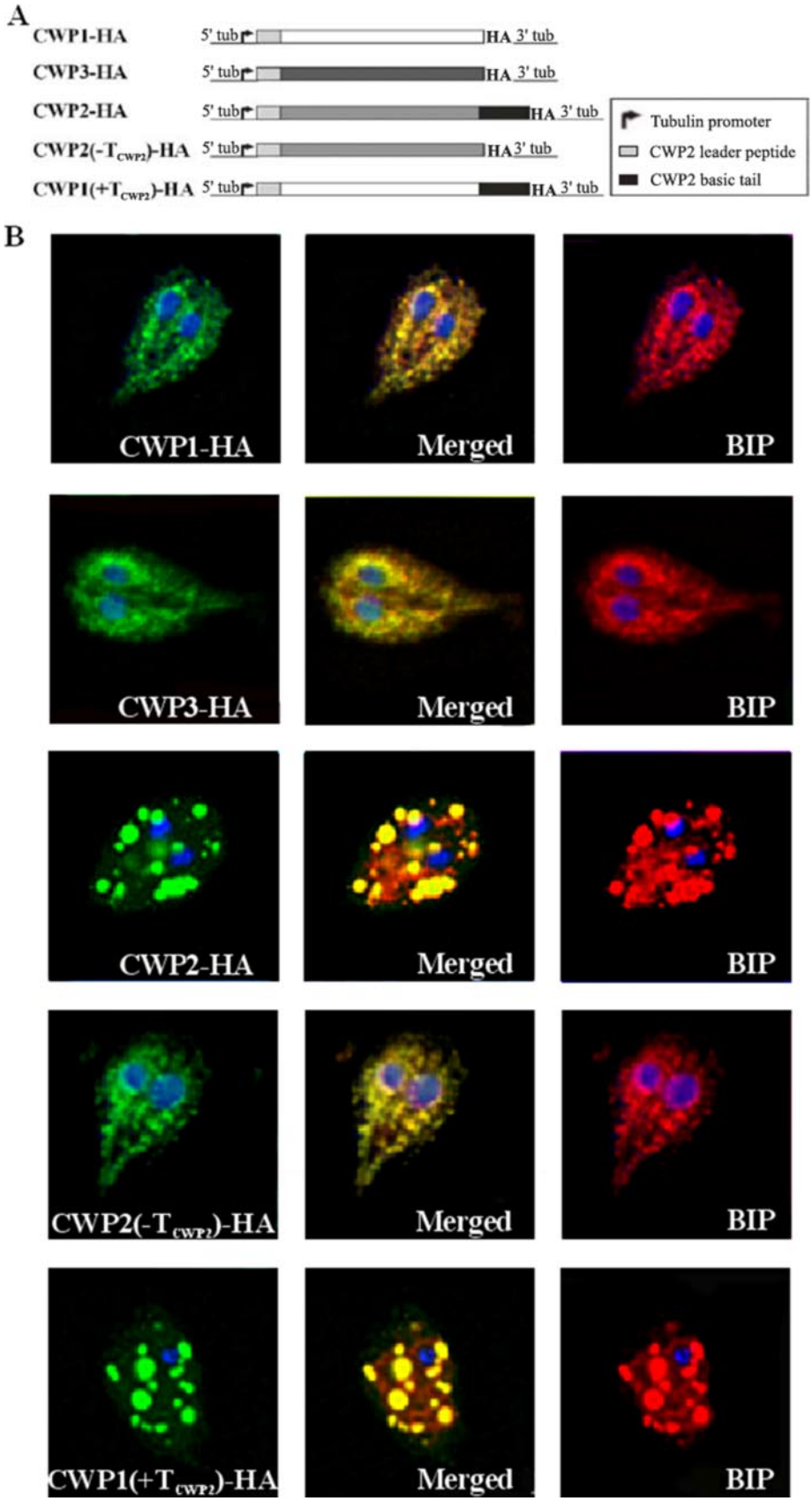

FIGURE 2. **Localization of transgenic CWPs in non-encysting trophozoites.** *A*, schematic representation of the different CWP constructs expressed in *Giardia*. *B*, confocal microscopy of direct immunofluorescence assays showing co-localization of CWP1-HA, CWP3-HA, and CWP2($-T_{CWP2}$)-HA (*green*) with BiP (*red*) to the ER (*yellow*). HA-tagged proteins were labeled with FITC-conjugated anti-HA mAb; BiP was labeled with Texas Red-conjugated mAb 9C9; and nuclei were visualized with DAPI (*blue*). Expression of CWP2-HA and CWP1($+T_{CWP2}$)-HA induced formation of secretory granules that also contained BiP. Magnification ×1000.

untransfected organisms (Figs. 1 and 3*B*) and were resistant to hypo-osmotic shock (data not shown), suggesting that the HA tag in CWPs does not interfere with cyst wall formation.

We also examined CWP expression by immunoblotting reduced and nonreduced protein extracts obtained from encysting and non-encysting *Giardia* trophozoites (Fig. 4). When extracts from cells expressing

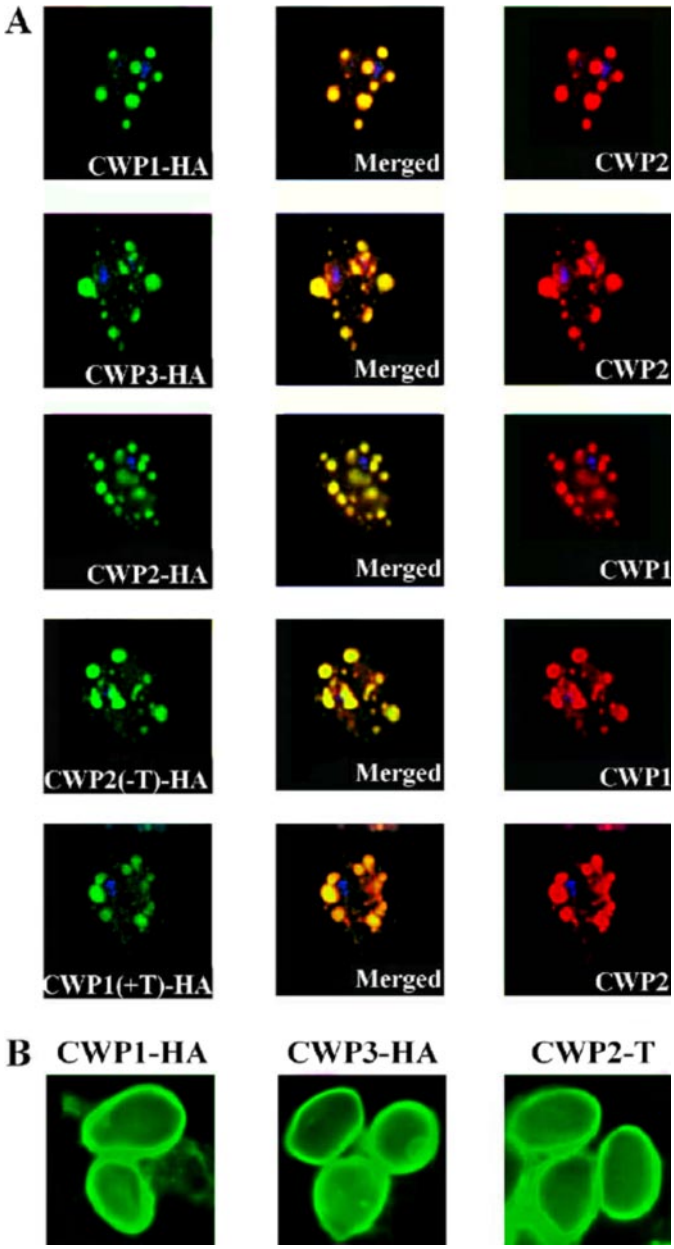

FIGURE 3. **Localization of different CWP variants in encysting *Giardia* trophozoites.** *A*, cells transfected with CWP1-HA, CWP3-HA, CWP2-HA, CWP2($-T_{CWP2}$)-HA, and CWP1($+T_{CWP2}$)-HA were cultured in encystation medium for 24 h and subjected to direct immunofluorescence analysis using FITC-conjugated anti-HA mAb (*green*) and either Texas Red-conjugated CWP2-specific mAb 7D2 or CWP1-specific mAb 5-3C (*red*). Confocal microscopy showed co-localization of the HA-tagged proteins with endogenous CWPs within the ESVs (*yellow*). The nuclei were stained with DAPI (*blue*). Magnification ×1000. *T*, T_{CWP2}. *B*, shown are the results from immunofluorescence analysis of transgenic cysts using anti-HA mAb (*green*). Magnification ×630.

CWP1-HA (Fig. 4*A*), CWP3-HA (Fig. 4*B*), and CWP2($-T_{CWP2}$)-HA (Fig. 4*C*) were reduced prior to electrophoresis, anti-HA mAb detected a band of ~26 kDa in non-encysting, pre-encysting, and encysting cells, corresponding to the correct molecular mass of non-aggregated proteins. In nonreduced samples from non-encysting and pre-encysting cells, an additional band of ~52 kDa potentially corresponding to the dimeric form was detected (Fig. 4, *A–C*). In encysting cells, we observed higher molecular mass species, including a clear ~65-kDa band, suggesting interaction of these 26-kDa CWPs with 39-kDa CWP2 (Fig. 4, *A–C*). Similarly, under reducing conditions in non-encysting and encysting cells, CWP2-HA and CWP1($+T_{CWP2}$)-HA expression revealed ~39-kDa species (Fig. 4, *D* and *E*), confirming the correct expression of these proteins. Under nonreducing conditions, the mAb detected an ~78-kDa band as well as other species and an upward smearing, which might represent proteins with variable degrees of glycosylation or cross-linking (2). In encysting cells, the smearing decreased under reducing conditions, suggesting CWP homo- or heterodimerization with other CWP family members. These data confirm previous results of sequential immunoprecipitation of different CWPs (7). Additionally, we confirmed CWP1-HA and CWP2-HA interaction with endogenous CWPs in encysting trophozoites by immunoprecipitation assays (supplemental Fig. 2).

Western blot analysis of spent culture medium from non-encysting trophozoites showed that CWPs lacking the CWP2 basic domain (CWP1-HA, CWP3-HA, and CWP2($-T_{CWP2}$)-HA) were constitutively secreted into the culture medium by non-encysting cells (Fig. 4*F*). Conversely, CWP2 and CWP1($+T_{CWP2}$)-HA, which are recruited into ESV-like vesicles, were not secreted into the medium (Fig. 4*F*). In these experiments, we utilized CWP-specific mAbs to bypass the potential HA tag loss due to low level encystation-specific cysteine protease activity in non-encysting cells (30).

Secretory Vesicles Induced in Non-encysting Trophozoites Have ESV Characteristics—Transmission electron microscopy showed that membrane-enclosed granules, induced by CWP2-HA and CWP1($+T_{CWP2}$)-HA expression in non-encysting trophozoites, are electron-dense, large, and rounded or irregular in shape like typical ESVs present in encysting cells (Fig. 5). Additionally, transgenic CWPs were present in membrane-enclosed compartments similar to the ESVs observed in encysting cells (supplemental Fig. 3). Moreover, ESV-like vesicles formed in transgenic non-encysting trophozoites could be isolated by subcellular fractionation similar to endogenous ESVs (supplemental Fig. 4).

CWP1 and CWP2 Co-localize in Secretory Granules in Non-encysting Trophozoites—In encysting trophozoites, CWP1-HA, CWP3-HA, and CWP2($-T_{CWP2}$)-HA were sorted to ESVs (Fig. 3*A*), suggesting that CWP2 acts as a cargo receptor directing other CWPs to the ESVs. To study whether CWP2 is involved in this process, we performed transient transfections with both CWP1-HA and CWP2-HA in non-encysting trophozoites. We determined their subcellular localization by using CWP2-specific mAb 7D2 and CWP1-specific mAb 5-3C. Direct double immunostaining of non-encysting trophozoites revealed several ESVs labeled with these mAbs, indicating that both CWP1-HA and CWP2-HA are within these granules and suggesting that CWP2 expression modifies CWP1 localization from the ER (Fig. 2*B*) to the ESV-like vesicles (Fig. 6*A*). When we performed similar experiments using CWP1-HA and CWP2($-T_{CWP2}$)-HA, both proteins co-localized in the ER (Fig. 6*B*), indicating the importance of the CWP2 basic extension in early ESV biogenesis. Negative immunostaining with mAb 9C3, which recognizes granule-specific protein (29), confirmed that these cells did not differentiate into cysts (data not shown). We verified the expression and molecular masses of HA-tagged CWP1 and CWP2 or HA-tagged CWP1 and CWP2($-T_{CWP2}$) by immunoblotting under reducing conditions (Fig. 6, *C* and *D*). Detection with anti-CWP1 mAb under nonreducing conditions in cells expressing CWP1-HA (26 kDa) and CWP2-HA (39 kDa) showed species corresponding to the molecular mass of the dimeric form of the CWP1 protein (~52 kDa, CWP1/CWP1) and a band representing the interaction between them (~65 kDa, CWP1/CWP2) (Fig. 6*C*, *left panel*). The same assay with anti-CWP2 mAb showed one band representing the interaction between the expressed CWPs (~65 kDa, CWP1/CWP2) and a band representing the interaction of CWP2 with itself (~78 kDa, CWP2/CWP2) (Fig. 6*C*, *right panel*). In control cotransfected cells, the two expressed proteins had the

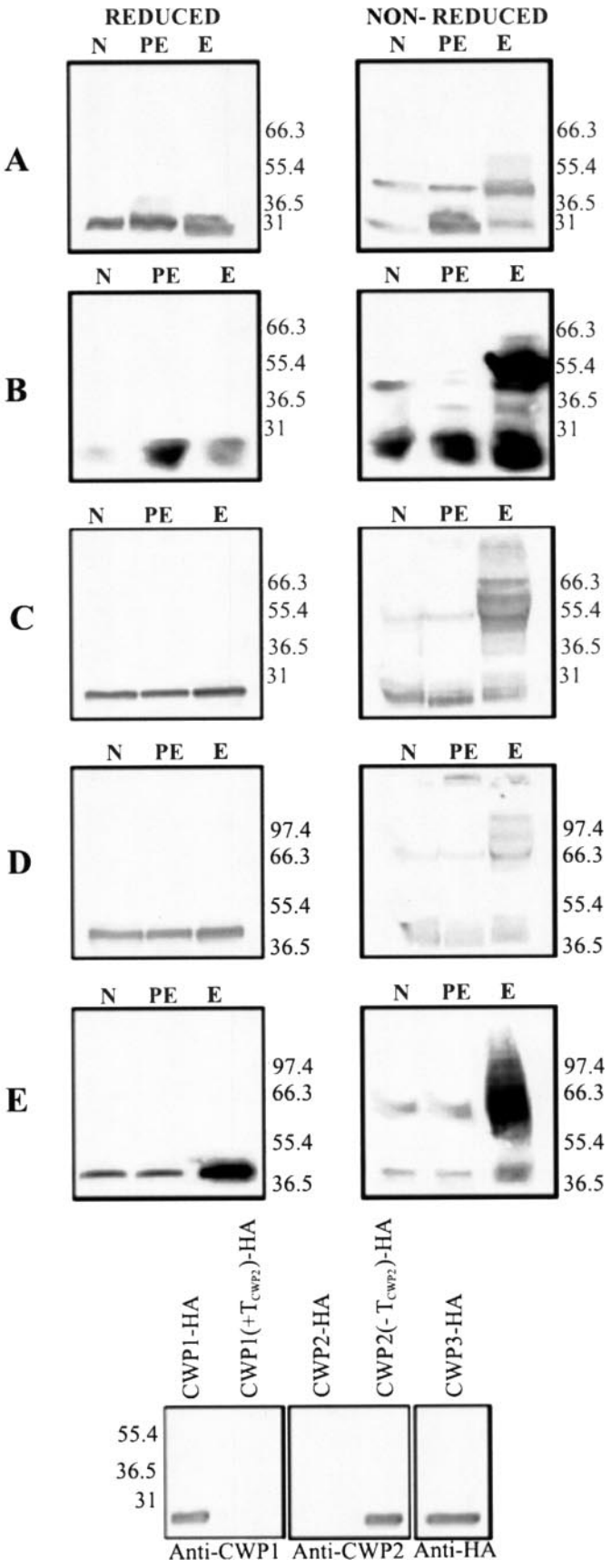

FIGURE 4. **Immunoblot and secretion assays of HA-tagged CWPs.** Trophozoites transfected with CWP1-HA (*A*), CWP3-HA (*B*), CWP2($-T_{CWP2}$)-HA (*C*), CWP2-HA (*D*), and CWP1($+T_{CWP2}$)-HA (*E*) were cultured in growth (*N*), pre-encystation (*PE*), or encystation (*E*) medium for 24 h. Immunoblot analysis of reduced (*left panels*) and nonreduced (*right panels*) total trophozoites using anti-HA mAb showed the correct expression of the HA-tagged proteins. Under nonreducing conditions, interaction of transgenic CWPs with themselves in non-encysting trophozoites or with other proteins in encysting trophozoites was observed. Secretion analysis of the transfected proteins was performed by immunoblotting the precipitated normal culture medium with mAb 5-3C for detection of CWP1 and CWP1($+T_{CWP2}$)-HA, mAb 7D2 for CWP2 and CWP2($-T_{CWP2}$)-HA, and anti-HA mAb for CWP3 (*F*).

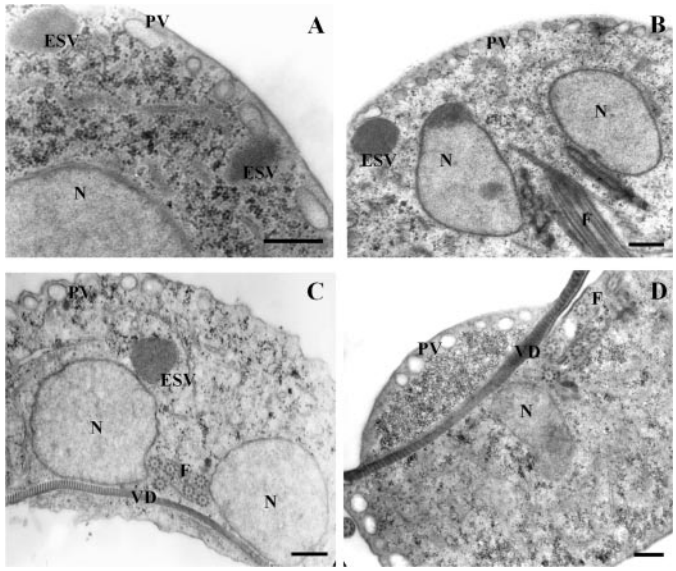

FIGURE 5. **Secretory granules generated by expression of CWP variants in non-encysting trophozoites are morphologically similar to ESVs in encysting cells.** Transmission electron analysis of ESVs generated in untransfected cells during encystation (*A*) or by transgenic expression of either CWP2-HA (*B*) or CWP1($+T_{CWP2}$)-HA (*C*) in non-encysting trophozoites showed that both types of vesicles share similar characteristics. Expression of CWP2($-T_{CWP2}$)-HA in non-encysting trophozoites did not result in formation of ESVs (*D*). *PV*, lysosome-like peripheral vesicles; *N*, nucleus; *F*, flagella; *VD*, ventral disk. *Scale bars* = 1 μm.

same molecular mass (26 kDa), and a band of ~52 kDa could be observed using both mAbs, which may correspond to the different possible interactions between the CWPs (CWP1/CWP1, CWP2-(T_{CWP2})/CWP2-(T_{CWP2}), and CWP1/CWP2-(T_{CWP2})) (Fig. 6*D*). CWP1 and CWP2 co-localization in these ESV-like vesicles and our previously reported co-immunoprecipitation studies (7) suggest that CWP heteroaggregation could be involved in segregating CWP1 into secretory granules.

The CWP2 Basic Extension Is Necessary but Not Sufficient to Induce Secretory Granule Biogenesis—To analyze whether the CWP2 basic domain is sufficient to trigger ESV biogenesis, we expressed HA-tagged versions of the CWP2 basic extension and chimeric variants of the surface protein VSPH7 in WB/1267 trophozoites. To express the HA-tagged version of the CWP2 basic extension (T_{CWP2}-HA), we placed the CWP2 signal peptide at the N terminus of the 13-kDa basic tail and the HA epitope at the C terminus of the polypeptide (Fig. 7*A*). The CWP2 basic extension localized predominantly to the ER in non-encysting trophozoites (Fig. 7*B*, *upper panels*) and in a clamp-shaped ER/nuclear envelope compartment in encysting cells (*lower panels*). In these structures, co-localization with endogenous CWPs was also observed (Fig. 7*B*, *lower panels*). Western blot analysis of culture supernatants from non-encysting trophozoites showed that the CWP2 basic domain was not secreted into the medium (Fig. 7*D*, *left panel*), suggesting that interaction with a receptor retains this polypeptide intracellularly.

VSPH7 is a variant-specific surface protein of *Giardia* clone GS/M-H7 (31). This protein possesses a leader peptide at the N terminus, a single transmembrane domain, and a conserved CRGKA cytoplasmic tail (Fig. 7*A*).0 VSPH7 is not expressed in *Giardia* clone WB/1267 (which expresses VSP1267), allowing detection of VSPH7 in transgenic tropho-

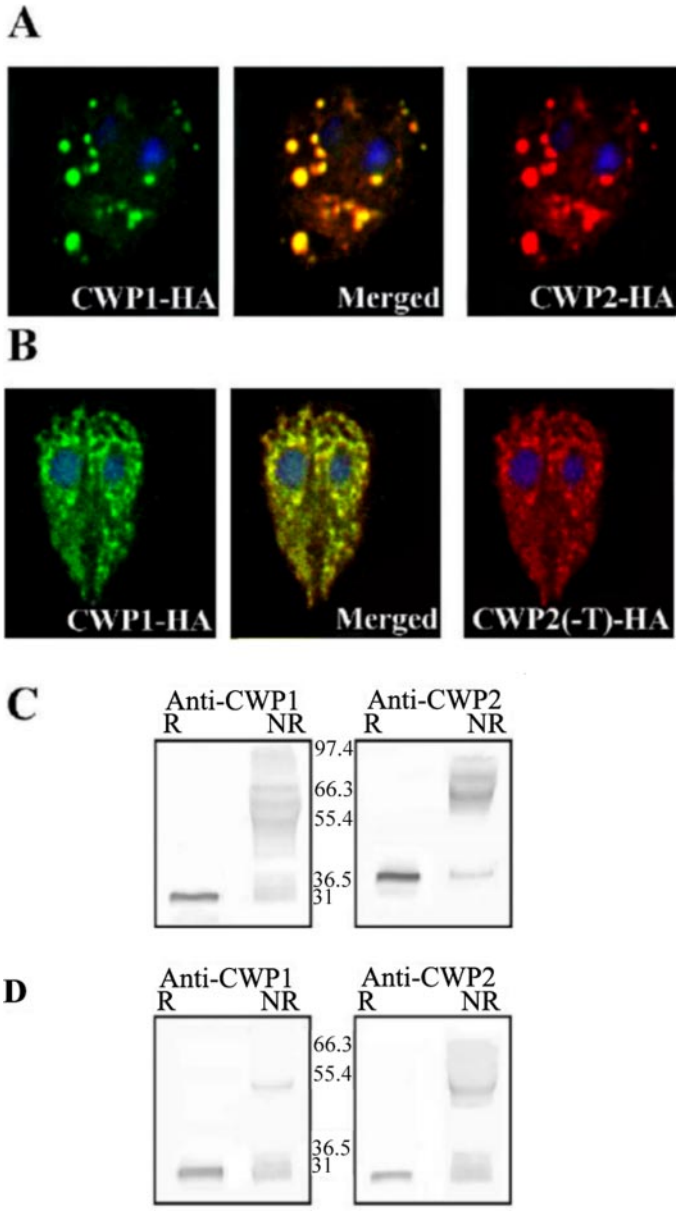

FIGURE 6. **Coexpression of CWP1 and CWP2 in non-encysting *Giardia* trophozoites.** *A*, confocal microcopy showed co-localization of CWP1-HA (*green*) and CWP2-HA (*red*) in transport vesicles (*yellow*) in non-encysting *Giardia* trophozoites expressing both proteins. *B*, when the same assay was performed in cells expressing CWP1-HA (*green*) and CWP2($-T_{CWP2}$)-HA (*red*), both proteins co-localized in the ER (*yellow*). Immunofluorescence analysis was performed using FITC-conjugated anti-CWP1 mAb 5-3C labeled and Texas Red-conjugated anti-CWP2 mAb 7D2. The nuclei were stained with DAPI (*blue*). Magnification ×1000. *T*, T_{CWP2}. *C* and *D*, immunoblot analysis was performed on non-encysting trophozoite protein extracts cotransfected with CWP1-HA/CWP2-HA and CWP1-HA/CWP2($-T_{CWP2}$)-HA, respectively, under reducing (*R*) and nonreducing (*NR*) conditions using anti-CWP1 and anti-CWP2 mAbs.

zoites using VSPH7-specific mAb G10/4, which recognizes the native conformation of the VSP (31). The sorting signal that directs VSPs to the plasma membrane is controversial. Evidence favors the transmembrane domain as a signal (18), but it has been suggested that the conserved CRGKA tail is also important (26). To analyze the role of the basic extension addition to this constitutively secreted protein, we constructed a chimera in which the basic extension is luminal. We removed the VSPH7 transmembrane domain (VSPH7(−TM)-HA) and added the CWP2 basic extension at the C terminus of this integral membrane protein (VSPH7($-TM+T_{CWP2}$)-HA) (Fig. 7*A*). The absence of the transmembrane domain altered VSPH7(−TM)-HA localization from the cell surface to the ER in both non-encysting and encysting cells (Fig. 7*C*, *left panels*). Western blot analysis detected this construct in the culture supernatant (Fig. 7*F*, *right panel*), indicating constitutive secretion. When the CWP2 basic tail was added to this modified VSP (VSPH7($-TM+T_{CWP2}$)-HA), it localized at the ER in non-encysting cells, but became more prominent around the nuclei in encysting cells (Fig. 7*C*, *right panels*), and the protein was not secreted (Fig. 7*D*, *right panel*). We observed the same pattern for VSP chimeras when VSPH7-specific mAb G10/4 was utilized (data not shown), indicating that these proteins are correctly folded. These results suggest that a signal present within the CWP2 extension is dominant over other regions of the VSP and that interactions between the CWP2 basic extension and a receptor cause retention within the ER.

Notably, expression of VSPH7 with a lumen-facing extension or the CWP2 basic extension in encysting cells reduced the number of granules/cell compared with untransfected trophozoites (Fig. 7*B*, *lower panels*). The average number of granules/cell was four and three for the encysting transfectant of VSPH7($-TM+T_{CWP2}$)-HA and T_{CWP2}-HA, respectively. The number of granules/cell for untransfected trophozoites was 13 ($n = 300$; $p < 0.001$ by paired *t* test).

Immunoblot analysis confirmed the protein expression. Under reducing and nonreducing conditions, HA-tagged molecules were present as single species at the correct molecular mass (Fig. 7, *E* and *F*), indicating that there is no interaction with other proteins through disulfide bonds.

DISCUSSION

In higher eukaryotes, the constitutive pathway functions to transport proteins to distinct organelles within the cell and to release molecules into the environment in a continuous manner, whereas the regulated pathway only discharges materials under the control of external stimuli (14). Proteins destined to the regulated pathway are sorted from those to be released via the constitutive pathway at the TGN and stored in secretory granules until their release is stimulated (14, 16). The early-branching protist *G. lamblia* possesses an interesting secretory system lacking an identifiable Golgi complex, and secretory granules are present only during trophozoite differentiation into cysts (19–21).

Because higher eukaryotic cells contain secretory granules during their entire life, the ability to regulate secretory granule formation in *Giardia* by changing the culture medium composition makes this parasite an exceptional model system (15, 32) to study how and where secretory granules form, how proteins are sorted and concentrated within these organelles, and why other proteins are excluded from them. The mechanisms of these processes remain under conjecture and need to be rigorously investigated (32).

In this work, we investigated the functional role CWPs in *Giardia* ESV biogenesis. CWP1, CWP2, and CWP3 have similar structures, but CWP2 differs at its C terminus because it contains a 13-kDa basic extension (pI 12.23), the function of which is unknown (6–8). Initially, we unsuccessfully attempted to knock down CWP expression in encysting trophozoites to determine the function of these proteins in ESV formation, similar to methods used to study other secretory proteins in *Giardia* (18, 29). To circumvent this problem, epitope-tagged CWPs were expressed individually or in combination in non-encysting trophozoites, a situation that is comparable with the knock-out methodology. In this life cycle stage, CWPs are not expressed. When we expressed CWP1, CWP3, and CWP2 lacking its basic extension in non-encysting cells, they entered the secretory pathway without forming granules and were secreted into the culture medium. Conversely, transgenic expression of CWP2 and

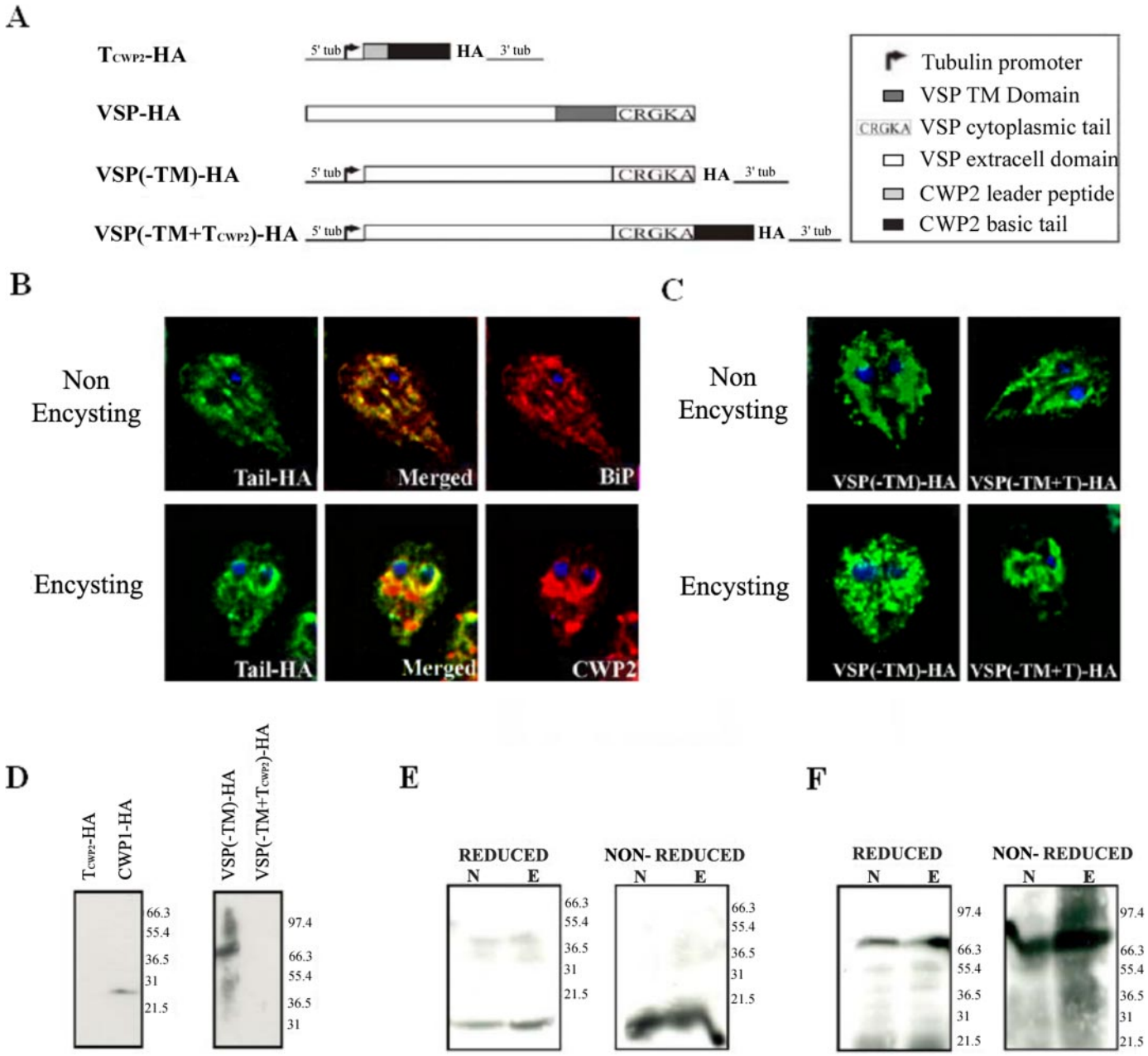

FIGURE 7. **Expression and localization of the basic tail of CWP2 and VSPH7 chimeras in non-encysting and encysting *Giardia* trophozoites.** *A*, schematic representation of the basic extension of CWP2, the VSPH7 protein, and the VSPH7 chimeras expressed in *Giardia* trophozoites. *tub*, tubulin. *B*, immunofluorescence assays of transgenic cells. HA-tagged proteins were stained with FITC (*green*), and nuclei were stained with DAPI (*blue*). Expressed T_{CWP2}-HA (*green*) co-localized with BiP (*red*) in the ER (*yellow*) in non-encysting trophozoites (*upper panels*) and in a clamp-shaped ER/nuclear envelope compartment with CWP2 (*red*) in encysting cells (*lower panels*). Magnification ×1000. *C*, indirect immunofluorescence assays of transgenic cells. HA-tagged proteins were stained with FITC (*green*), and nuclei were stained with DAPI (*blue*). Expressed VSPH7(−TM)-HA and VSPH7(−TM+T_{CWP2})-HA localized in the ER in non-encysting cells (*upper panels*). In encysting cells, VSPH7(−TM)-HA also localized in the ER, and VSPH7(−TM+T_{CWP2})-HA localized in a clamp-shaped ER/nuclear envelope compartment (*lower panels*). Magnification ×1000. *D*, secretion analysis of the transfected T_{CWP2}-HA, VSPH7(−TM)-HA, and VSPH7(−TM+T_{CWP2})-HA proteins in encysting cells immunoblotted from the precipitated culture medium with anti-HA mAb. *E*, immunoblot analysis of total protein extract of trophozoites transfected with T_{CWP2}-HA cultured in growth (*N*) or encystation (*E*) medium for 24 h under reducing (*R*) and nonreducing (*NR*) conditions. *F*, immunoblot analysis of total protein extract of trophozoites transfected with VSPH7(−TM+T_{CWP2})-HA cultured in growth or encystation medium for 24 h under reducing and nonreducing conditions.

CWP1 plus the CWP2 basic extension in non-encysting trophozoites induced the biogenesis of large secretory granules that shared common electron density and microscopic characteristics with the typical ESVs that normally transport cyst wall materials during *Giardia* encystation. These granules never discharged their contents to the cell exterior, and no cyst wall was formed in transgenic non-encysting trophozoites. These data agree with previous reports showing that additional proteins are required for secretion and cyst wall assembly. For example, ESV exocytosis requires encystation-specific cysteine protease activity to cleave the CWP2 basic extension (30) and granule-specific protein to regulate granule discharge during exocytosis (29). These results demonstrate that the CWP2 basic extension is necessary for ESV biogenesis during the regulated pathway.

Additional results show that expression of the CWP2 basic extension alone or the chimeric protein VSPH7(−TM+T_{CWP2})-HA did not induce ESV biogenesis and that these constructs localized predominantly to the ER in non-encysting cells. Because the basic

extension alone is not sufficient to induce ESV biogenesis, other CWP2 domains are likely necessary for this process. The leucine-rich repeats and the cysteine-rich region of CWP2 are potential candidates for this activity because they are known to be involved in protein/protein interactions (10). In immunoblot analyses of nonreduced protein samples, CWPs formed self-aggregates in non-encysting cells or interacted with other CWPs in encysting trophozoites. In contrast, T_{CWP2}-HA and VSPH7($-$TM$+T_{CWP2}$)-HA were not sorted to the ESVs and did not form aggregates under the same conditions, suggesting that CWP aggregation is also necessary for ESV biogenesis. Thus, our results propose that interactions between CWP1 and CWP3 with CWP2 are also needed for ESV biogenesis. These data agree with previous research highlighting the importance of different CWP domains in targeting CWP1 and CWP3 to ESVs during encystation (8, 9, 23). Similar to Weibel-Palade body biogenesis in endothelial cells (33), high molecular mass multimer formation *per se* is not sufficient to trigger secretory granule formation; an active sorting step is also required. In addition, coexpression of CWP1-HA and CWP2-HA in non-encysting trophozoites changed CWP1-HA localization from the ER to ESV-like vesicles, probably as a result of its interaction with CWP2. This finding is similar to what occurs in mammalian cells, in which it was demonstrated that chromogranins A and B, because of their tendency to associate with each other in a pH- and Ca^{2+}-dependent manner, can form stable aggregates, acting as a physical driving force for granulogenesis in the TGN (34, 35).

Interestingly, ESVs induced by expression of CWP2-HA or CWP1($+T_{CWP2}$)-HA co-localized with endogenous CWPs (Fig. 3). If overexpression of these proteins causes nonspecific protein aggregates, they should likely appear in different structures when labeled with anti-HA mAb.

Our results implicate CWP2 as an aggregation nucleation point for other CWPs destined to the ESVs. If true, interaction between the basic extension and an anionic receptor could tether CWP2 to segregation compartment membranes. Subsequent CWP interactions with CWP2 could lead to complex formation and secretory granule budding. Thus, this compartment could effectively act as a *trans*-Golgi, excluding proteins from ESVs not destined to these granules. Many proteins contain basic amino acid clusters that can bind to acidic phospholipids, which are preferentially located on the luminal cell membrane surface or on acidic proteins (36, 37). The microneme and dense granule proteins of *Plasmodium*, *Babesia*, and *Toxoplasma* (38–40) also contain basic amino acid clusters. Therefore, the mechanism described here might be more globally applicable with regard to secretory granule biogenesis in other relevant parasitic protozoa.

Supporting this idea is that a receptor in luminal membranes is indispensable for secretory granule formation. We observed in encysting trophozoites expressing T_{CWP2}-HA or VSPH7($-$TM$+T_{CWP2}$)-HA that the ESV number was reduced. Possibly in these cases, the CWP2 basic extension competes with endogenous CWP2 for an anionic receptor. Localization of T_{CWP2}-HA and VSPH7($-$TM$+T_{CWP2}$)-HA moved from the ER to a more limited clamp-shaped pattern adjacent to the ER/nuclear envelope compartment, in a pattern similar to the Golgi-specific marker 12-(*N*-methyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl))-ceramide (3, 41). In these experiments, the presence of the basic extension inhibited extracellular secretion of chimeras containing the CWP2 basic extension. Retention of these expressed proteins in that specific compartment led us to speculate that the intracellular receptor for the basic extension is localized in a region near the nuclei in encysting trophozoites. This specialized compartment is derived from the ER because blockage of ER exit at 15 °C inhibits formation of ESVs both in encysting trophozoites (supplemental Fig. 5) (26) and in transgenic non-encysting trophozoites expressing CWPs containing the CWP2 basic domain (supplemental Fig. 5). Moreover, coexpressed CWP1 and CWP2 lacking its basic extension co-localized to the ER. However, biogenesis of ESVs was not induced, suggesting that interaction among CWPs is not sufficient to trigger ESV formation. A sorting receptor is likely necessary to segregate CWPs into the specific compartments that will then generate an ESV. We have been unsuccessful in isolating potential receptor candidates using T_{CWP2}-HA in pulldown or yeast two-hybrid assays, probably because of either the strong basic character of CWP2 or the potential receptor's lipid characteristics. Transgenic CWP2 expression in AtT-20 and COS-7 cells (cells that do and do not have a regulated secretory pathway, respectively) did not induce the formation or sorting of CWPs to secretory granules (data not shown). This suggests either that these cells lack the protein receptor or that their membranes have a different lipid composition. Expression of heterologous protein in animal cells often results in its retention in the ER. In our case, CWPs were constitutively secreted and found in the culture supernatant (data not shown). These data agree with recent work demonstrating that both CWP1 and CWP2 are targeted to transport vesicles in HEK293 cells; these cells have a prominent constitutive pathway, but lack a regulated secretory pathway and secrete without inducing secretory granule formation (42).

The traditional model for regulated secretion proposes four distinct events (15): 1) selective condensation or precipitation of the proteins that aggregate to form a dense core, 2) selection of membranes that envelop the aggregate, 3) fusion/fission of the membrane to release the nascent secretory granule, and 4) granule maturation. The first step is believed to be selective because of the intrinsic properties of the regulated proteins packaged in the cores. The second step may be mediated by a specific receptor or receptors in the TGN membrane that bind aggregated secretory proteins. Carboxypeptidase E, an enzyme involved in the conversion of prohormones, has been proposed to be the sorting receptor in all endocrine cells (43), but this finding remains an open question (44). Moreover, studies have indicated that aggregated proteins interact directly with lipid membrane constituents (*e.g.* cholesterol) and that this interaction leads to reorganization of cholesterol-rich microdomains and immature granule budding (45).

An additional model for secretory granule formation derives from examining prolactin-producing cells (46). Granules form when the *trans*-Golgi layer is entirely consumed by small vesicle budding, leaving behind membrane-enclosed aggregates. These become secretory granules that progressively mature as small vesicle budding proceeds. Although such a mechanism clearly occurs in mammary gland cells, how secretory granule biogenesis occurs in other eukaryotic cells is not well understood (32).

The traditional model seems applicable in *G. lamblia* because the intrinsic characteristics of the CWPs, in addition to the requirement of CWP2 basic extension interaction with a receptor, are important for ESV biogenesis. On the basis of our results, we propose, however, that membrane selection is the initial step in secretory granule biogenesis. Because the synthesis of one receptor for each protein destined to granules or a unique receptor for all is highly unlikely, our results support the possibility that the elusive sorting receptor necessary for sorting proteins to secretory granules in eukaryotes may well be lipid molecules (45).

Proteins that are able to form large aggregates may condense earlier in the secretory pathway, not just in the *trans*-Golgi (47, 48). In *Giardia*, the concentration of CWP1, CWP2, and CWP3 in heteroaggregates

previously sorted by the CWP2 basic extension drives secretory granule biogenesis. The dense granules formed by CWP aggregation might then mature by further addition of CWPs and other granule components (2, 3). The up-regulation observed for *Giardia* BiP (23) and protein-disulfide isomerase (49) during encystation and their presence at the ESVs (23, 50) suggest that chaperones can assist CWP folding and CWP complex formation. These might be necessary to maintain CWPs in a stable form within the ESVs, inhibiting an early and unproductive polymerization of cyst wall components (2). Supporting this possibility is the fact that the chaperone Cpn60 is present in immature secretory granules of insulin-secreting cells and is involved in insulin processing and packaging (51). Because CWP exit from the ER is necessary for ESV biogenesis (supplemental Fig. 5), the presence of BiP at later steps of the regulated secretory pathway (Figs. 2–4) is an indication that CWP sorting to the ESVs occurs directly from an ER-derived compartment. Interestingly, transport of plant storage proteins to the vacuole is also thought to take place from the ER (52). In seeds, protein bodies formed by aggregation of storage proteins contain BiP and protein-disulfide isomerase, suggesting that the Golgi apparatus is not involved in the transport of these proteins to the vacuole (52, 53).

Marti and Hehl (54) proposed that ESVs develop by small vesicle association and that ESVs mature by retrograde transport to the ER, followed by complete dispersal of ESVs into small secretory vesicles before cyst wall protein secretion. They speculated that ESVs could correspond to Golgi cisternae, which may represent the TGN of eukaryotic cells (55). More recently, the same group found BiP in a proteomic analysis of purified ESVs, supporting their claim that ESVs are Golgi equivalents (24). However, their proteomic analysis failed to find several known granule components such as CWPs and granule-specific protein, but identified different subunits of the proteasome. (In that particular work, no information regarding the quality of the ESV preparation was presented.)

On the basis of our results, we propose an alternative scenario in which ESVs are formed by CWP aggregation with the later budding of these granules in a compartment that could be a specialized ER or Golgi-like organelle. This view is supported by data from detailed electron analysis of encysting cells with ESVs arising from modified ER cisternae (22). These cells have large ESVs that are not fragmented before exocytosis, and labeling of encysting trophozoites with different Golgi markers (12-(*N*-methyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl))-ceramide or antibodies against ADP-ribosylation factor and β-coat protein showed no staining of ESVs (3, 22, 56). Conversely, Hehl and co-workers (24) showed that polyclonal antibodies generated against *Giardia* β-coat proteinand Yip1p are able to label ESVs that contain CWP2. They also reported that an antibody to clathrin labels ESVs, but they failed to find this molecule in purified granules (24, 55). Besides, Yip1p is known to cycle between the ER and the Golgi apparatus and is not considered a *bona fide* Golgi marker (57). In *Giardia*, expression in encysting or non-encysting trophozoites of HA-tagged p115 (a vesicle-docking protein essential for biogenesis of the Golgi apparatus in mammalian cells) (58), the *Giardia* KDEL receptor (20), or mammalian galactosyltransferase 2 (a mammalian glycosyltransferase) (59) showed that these proteins localized in perinuclear structures and not in ESVs.[3] Taken together, these data provide compelling evidence against the view that the electron-dense ESVs are transient Golgi cisternae instead of providing evidence for *de novo* ESV biogenesis during encystation in a *Giardia* compartment derived from the ER.

In summary, we have provided results showing the molecular basis for secretory granule biogenesis in *Giardia*, which might allow not only the development of cyst formation inhibitors and mechanisms for controlling the dissemination of the disease but also provide new clues about the evolution/involution of the eukaryotic secretory pathway. Because *Giardia* is one of the earliest branching protists, knowledge of the secretory organelle biogenesis that occurs during its differentiation into cysts offers novel insights into the molecular machinery required for regulated protein transport in higher organisms. Although *Giardia* may have lost "essential" genes/organelles because of its parasitic life style, its study can also facilitate the comprehension of the secretory machinery of many other important human parasites.

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[3] N. Gottig and H. D. Luján, unpublished data.

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