The nuclear envelope serves as an intermediary between the ER and Golgi complex in the intracellular parasite *Toxoplasma gondii*

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SUMMARY

Morphological examination of the highly polarized protozoan parasite *Toxoplasma gondii* suggests that secretory traffic in this organism progresses from the endoplasmic reticulum to the Golgi apparatus using the nuclear envelope as an intermediate compartment. While the endoplasmic reticulum is predominantly located near the basal end of the parasite, the Golgi is invariably adjacent to the apical end of the nucleus, and the space between the Golgi and nuclear envelope is filled with numerous coatomer-coated vesicles. Staining with antiserum raised against recombinant *T. gondii* β-COP confirms its association with the apical juxtanuclear region. Perturbation of protein secretion using brefeldin A, microtubule inhibitors or dithiothreitol disrupts the Golgi, causing swelling of the nuclear envelope, particularly at its basal end. Prolonged drug treatment leads to gross distention of the endoplasmic reticulum, filling the basal end of the parasite. Cloning and sequencing of the *T. gondii* homolog of the chaperonin protein Bip identifies the carboxy-terminal amino acid sequence HDEL as this organism’s endoplasmic reticulum-retention signal. Appending the HDEL motif to a recombinant secretory protein (a chimera between the parasite’s major surface protein fusion, P30, and the Green Fluorescent Protein) causes this secretory reporter to be retained intracellularly. P30-GFP-HDEL fluorescence was most intense within the nuclear envelope, particularly at the apical end. These data support a model of secretion in which protein traffic from the endoplasmic reticulum to Golgi occurs via the apical end of the nuclear envelope.

Key words: Secretory pathway, Polarized secretion, Apicomplexan parasite, *Toxoplasma gondii*

INTRODUCTION

The phylum Apicomplexa includes *Plasmodium* (the causative agent of malaria), *Theileria* (an important cattle pathogen), *Toxoplasma* (a prominent opportunistic infection associated with AIDS) (McLeod and Remington, 1987), and approximately 5000 other named species (Levine, 1988). Certain apicomplexans are among the smallest eukaryotes known, <1 μm in diameter (Chobotar and Scholtyseck, 1982). One can ask of these organisms: what is the minimum equipment necessary to make a functional cell, and how is that basic equipment elaborated as the parasite grows, divides and infects other cells? Remarkably, many smaller apicomplexans (e.g. *Plasmodium, Theileria*) possess no morphologically distinctive Golgi apparatus (Shaw and Tilney, 1992; Ward et al., 1997), despite their need to properly target proteins to various specialized organelles and to secrete the materials necessary for host cell invasion and intracellular survival (Dubremetz, 1998; Lingelbach and Joiner, 1998).

In contrast to the highly reduced organization of *Plasmodium* and *Theileria*, *Toxoplasma*, a banana-shaped parasite somewhat larger in size (approx. 2 × 8 μm), exhibits a classic eukaryotic morphology, with readily recognizable nucleus, mitochondrion, ER, Golgi apparatus, etc. (Chobotar and Scholtyseck, 1982). Poised midway between minute organisms with poor ultrastructural resolution (e.g. *Plasmodium, Theileria, Saccharomyces*) and the morphological complexity of mammalian cells (for example), the entire *T. gondii* secretory pathway can be visualized within a few thin sections under the electron microscope.

*Toxoplasma* is a ubiquitous parasite, capable of infecting virtually any nucleated cell (Dubremetz, 1998). Within these cells, parasite ‘tachyzoites’ replicate rapidly, inside a specialized parasitophorous vacuole. Host cell invasion, establishment of the parasitophorous vacuole, and intracellular survival are thought to require the secretion of numerous parasite proteins from the rhoptries and micronemes (a complex of specialized apical organelles that gives the phylum Apicomplexa its name) (Carruthers and Sibley, 1997; Dubremetz, 1998). Additional secretory vesicles (termed dense granules) release materials believed to maintain the parasitophorous vacuole, and may also deliver proteins that modify the vacuolar environment (Cesbron-Delauw et al., 1996; Karsten et al., 1998; Lingelbach and Joiner, 1998). Understanding the nature of
secretion in *T. gondii* will therefore be critical for elucidating (and interfering with) parasite invasion and intracellular survival.

*T. gondii* tachyzoites are highly polarized cells (Chobotar and Scholtysiek, 1982). The central region of the parasite is dominated by the nucleus, effectively dividing the cytoplasm into basal and apical domains (Ward et al., 1997). Most of the ER is found in the basal cytoplasm, while the apical cytoplasm contains the Golgi and specialized secretory organelles noted above. These observations suggest that secretory transit from the ER to Golgi occurs via the nuclear envelope. In this report, we have sought to test this hypothesis using a combination of morphological, immunological, pharmacological and molecular approaches.

**MATERIALS AND METHODS**

**Cells, parasites and drugs**

Primary human foreskin (HFF) cells were grown in Modified Eagle’s Medium (Gibco, Grand Island, NY) containing 10% heat-inactivated, iron-supplemented newborn bovine serum (Hazleton Biologics, Lenexa, KS), as previously described (Roos et al., 1994). For microscopy, 10^5 cells were inoculated directly into 35 mm Petri dishes containing 25 mm diameter #1 glass coverslips. Cells were maintained as a contact-inhibited monolayer until required. The RH strain of *T. gondii* was maintained by serial passage in HFF cells grown in 25 cm^2 T-flasks. For infection, culture dishes were inoculated with freshly harvested tachyzoites and incubated in Minimal Essential Medium containing 1% fetal bovine serum (Gibco). Cultures were infected at a multiplicity of ~1 infectious unit/cell) 12-24 hours prior to microscopy, 10^5 cells were inoculated directly into 35 mm Petri dishes containing 25 mm diameter #1 glass coverslips. Cells were maintained as a contact-inhibited monolayer until required. The RH strain of *T. gondii* was maintained by serial passage in HFF cells grown in 25 cm^2 T-flasks. For infection, culture dishes were inoculated with freshly harvested tachyzoites and incubated in Minimal Essential Medium containing 1% fetal bovine serum (Gibco). Cultures were infected at a multiplicity of ~1 infectious unit/cell) 12-24 hours prior to experimental manipulation. As tachyzoites replicate synchronously within the specialized intracellular vesicles in which they reside, this procedure yields numerous parasitophorous vacuoles containing 2-16 parasites.

Brefeldin A was obtained from Sigma (St Louis, MO), dissolved in dimethylsulfoxide (DMSO) at 5 mg/ml, and samples stored frozen at −80°C until needed. Infected HFF cells were incubated in brefeldin A at a concentration of 20 μg/ml. Ethalfluralin (N-ethyl-N-(2-methyl-2-propenyl)-2,6-dinitro-4-trifluoromethylzenamine) and other dinitro-compounds were synthesized by Dr J.W. Benbow (Lehigh University, Bethlehem, PA) and Dr Randy Schekman (Bednarek et al., 1995). To produce anti-*T. gondii* β-COP and anti-*T. gondii* Sec23p, the partial coding sequences described above were excised (β-COP) or PCR-amplified (Sec23p) as BamHI-Sall fragments, and ligated into E. coli expression vector pGEX-5X-3 (Pharmacia Biotech, Inc.). Recombinant GST fusion proteins of the predicted sizes (Tgβ-COP: 22 kDa+30 kDa GST=52 kDa; TgSec23p: 29 kDa+30 kDa GST=59 kDa) were expressed and purified using GS beads according to the manufacturer’s recommended protocol, concentrated and stored at −80°C. Purity was assayed by SDS-PAGE and concentration determined by absorbance at 280 nm. 100 μg recombinant fusion protein in complete Freund’s adjuvant was used to immunize previously *T. gondii*-seronegative New Zealand White rabbits by intramuscular injection, followed by boosts with 50 μg protein (in incomplete Freund’s) at weeks 2, 3 and 6 (Cocalico Biologicals Inc., Reamstown PA). Western analysis identified two parasite proteins, approximately 100 kDa and 220 kDa in size, that reacted with anti-*T. gondii* β-COP antiserum, and one approx. 80 kDa protein that reacted with anti-Sec23p.

**Cloning of *T. gondii* secretory pathway proteins and engineering of recombinant secretory reporters**

Several *T. gondii* sequences exhibiting strong sequence similarity to secretory pathway proteins from other species were identified as expressed sequence tags (Ajikata et al., 1998; http://www.cbil.upenn.edu/ParaDBs/). EST clone #513215 (encoding *T. gondii* BiP) was excised, sequenced and used to screen a *T. gondii* cDNA library (AIDS Reference and Reagent Repository, NIH). Several full-length clones containing a 1.9 kb cDNA were identified and fully sequenced. EST clone #623080 (kindly provided by Dr David Sibley) exhibited strong similarity to β-COP, and the 1.7 kb cDNA was excised and sequenced to reveal an open reading frame encoding the C-terminal 200 approx. amino acids of *T. gondii* β-COP. Similarly, EST clone #467453 exhibited strong similarity to Sec23p, and the 1.3 kb cDNA was excised and sequenced to reveal an open reading frame encoding the C-terminal 200 approx. amino acids of *T. gondii* Sec23p. Full-length TgBiP and partial Tgβ-COP and TgSec23p sequences are available from GenBank, with accession numbers AF110397, AF163574, and AF163575 (respectively). No effort has been made to obtain full-length cDNA clones encoding Tgβ-COP or TgSec23p.

The putative ER retention motif of BiP (Monro and Pelham, 1987) was identified as HDEL, and engineered onto the C terminus of secretory reporter P30-GFP (Striepen et al., 1998) by PCR, using a sense primer upstream of the GFP coding sequence and antisense primer 5’-ctgcagctcagCTACAACCTCGTGTGTTGTATAGTCTACATGCATCAT-3’ (underlined nucleotides introduce the HDEL motif in antisense orientation; stop codon shown in bold; italics indicate the PrI site used for cloning). The resulting AvrII-PrI fragment was used to replace the entire GFP domain in plasmid P30-GFP, which contains a signal sequence derived from the major surface antigen of *T. gondii* (Burg et al., 1991).

**Antibodies**

Heterologous antisera were kindly provided by Drs Jay Bangs (anti-*Trypanosoma brucei* BiP; Bangs et al., 1996), Jennifer Lippincott-Schwartz (anti-β-COP, prepared against the ‘EAGE’ sequence; Duden et al., 1991) and Randy Schekman (Bednarek et al., 1995). To produce anti-*T. gondii* β-COP and anti-*T. gondii* Sec23p, the partial coding sequences described above were excised (β-COP) or PCR-amplified (Sec23p) as BamHI-Sall fragments, and ligated into E. coli expression vector pGEX-5X-3 (Pharmacia Biotech, Inc.). Recombinant GST fusion proteins of the predicted sizes (Tgβ-COP: 22 kDa+30 kDa GST=52 kDa; TgSec23p: 29 kDa+30 kDa GST=59 kDa) were expressed and purified using GS beads according to the manufacturer’s recommended protocol, concentrated and stored at −80°C. Purity was assayed by SDS-PAGE and concentration determined by absorbance at 280 nm. 100 μg recombinant fusion protein in complete Freund’s adjuvant was used to immunize previously *T. gondii*-seronegative New Zealand White rabbits by intramuscular injection, followed by boosts with 50 μg protein (in incomplete Freund’s) at weeks 2, 3 and 6 (Cocalico Biologicals Inc., Reamstown PA). Western analysis identified two parasite proteins, approximately 100 kDa and 220 kDa in size, that reacted with anti-*T. gondii* β-COP antiserum, and one approx. 80 kDa protein that reacted with anti-Sec23p.

**Transient and stable parasite transformation**

Chimeric *dhfr*-P30-GFP-HDEL/Sag-CAT plasmids (Striepen et al., 1998) were transfected into *T. gondii* parasites as described previously (Roos et al., 1994) using 10^7 freshly harvested tachyzoites and 50 μg sterilized plasmid DNA in a 2 mm gap cuvette (BTX; 1.5 kEV pulse, resistance 24 Ω). After electroporation, parasites were inoculated into HFF cell cultures. Transiently transfected samples were viewed 18-24 hours post-transfection. Stable transfectants were selected for chloramphenicol resistance and cloned by limiting dilution in 96-well plates.

**Microscopy**

For immunofluorescence, infected cells grown on glass coverslips were rinsed in PBS, fixed for 5 minutes in 2% formaline (pH 7.4), and permeabilized for 5 minutes in 0.25% Triton X-100 (at room temperature) or 2 minutes in methanol (at −20°C). Following several washes in PBS, samples were blocked for 30-60 minutes in 10% fetal bovine serum (or 10% non-fat milk powder), inverted over 50 μl primary antibody solution (diluted 1:500 in PBS), and maintained in a humidified chamber at room temperature for 60-90 minutes. After additional washing in PBS, coverslips were incubated with FITC-conjugated goat anti-rabbit secondary antibody (Sigma). Control samples of uninfected cells or primary or secondary antibody alone were also examined. Coverslips were mounted on glass slides using a minimal volume of Fluormount G (Southern Biotechnology Associates, Birmingham AL), and viewed using standard FITC excitation/barrier filters (450-480/515-565 nm) on a Zeiss IM-35 Axioscope illuminated with a mercury lamp, or a Nikon Optiphot equipped with confocal imaging equipment (Kr/Ar laser, BioRad
MRC-600 scanning system, COMOS software; BioRad). GFP fluorescence was detected in living cells, with imaging as above.

For electron microscopy, infected cultures in 35 mm dishes were fixed in situ by carefully replacing the culture medium with a freshly prepared solution of 1% glutaraldehyde (8% stock; Electron Microscope Sciences, Fort Washington, PA) and 1% OsO4 in 50 mM phosphate buffer (pH 6.3). Fixative was added at room temperature and incubation carried out on ice for 45 minutes, after which samples were rinsed in distilled water to remove excess phosphate, stained en bloc in 0.5% uranyl acetate overnight, dehydrated, and embedded in Epon directly in the original Petri dishes. Once the plastic had cured, specimens were popped out of their dishes, reoriented as required and trimmed. Thin sections were cut with a diamond knife, picked up on uncoated grids, stained with uranyl acetate and lead citrate, and examined using a Phillips 200 electron microscope (Phillips Electronic Instruments; Mahwah, NJ).

RESULTS

Compartments of the Toxoplasma secretory pathway

The nucleus of T. gondii tachyzoites is centrally located, effectively dividing the cytoplasm into apical and basal domains (Fig. 1). The single prominent Golgi apparatus is invariably found closely associated with the anterior end of the nucleus, often within a cup-like flattening or indentation. This association is maintained throughout the parasite cell cycle (as is the case in many protozoa, the nuclear envelope does not break down during mitosis). The rough ER can be found throughout the cytoplasm, but is concentrated at the basal end, posterior to the nucleus. The ER is tubular in form, and favorable sections demonstrate continuity with the basal and lateral surfaces of the nuclear envelope (arrow in Fig. 1A). Although we have not carried out extensive reconstructions, serial sections suggest that even when ER elements are observed in the apical end of the parasite, they are connected to the nuclear envelop via ‘horns’ extending apically from the lateral side of the nucleus (Fig. 1B). The ER never makes direct contact with the anterior surface of the nuclear envelope, which is the region adjacent to the Golgi.

Closer examination of the T. gondii Golgi complex reveals that its ‘cis’ side consists of a row of vesicles that appear to be pinching away from (or fusing with) the outer nuclear envelope (Fig. 1C). A thin coat covers the surface of these vesicles. Immediately anterior to this row of vesicles lies a stack of 3-6 flattened cisternae comparable to the cis-, medial- and trans-Golgi of better-known eukaryotes (Mellman and Simons, 1992; Palade, 1975), although no functional data is yet available to define these compartments in biochemical terms. Another type of coated vesicle, morphologically similar to clathrin-coated vesicles, is frequently found along the lateral margins of the trans-Golgi, and clathrin baskets can be visualized by electron microscopy of ruthenium red-stabilized parasites (not shown). Some of these vesicles enclose dense matter similar in staining characteristics to the material present in developing rhoptries.

This basic morphology suggests that Toxoplasma might be using the nuclear envelope as an intermediate compartment between the ER (attached to its basal and lateral surfaces), and the Golgi complex, immediately adjacent to its apical end. Such an organization would facilitate vectorial secretory

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Fig. 1. Ultrastructure of the Toxoplasma gondii secretory apparatus. (A) Thin section electron micrograph of an intracellular T. gondii tachyzoite. Only a single Golgi apparatus is present in each parasite cell, and this is closely apposed to the anterior pole of the nucleus. Extensions of rough ER project from the basal and lateral margins of the nucleus (arrow). Even at this magnification, vesicles can be seen budding from the outer nuclear envelope in the Golgi region. The large elongated structures are rhoptries, and the small structures microsomes; both are thought to act as secretory vacuoles during host cell penetration (Carruthers and Sibley, 1997; Dubremetz, 1998). Bar, 0.5 μm. (B) The secretory equipment of T. gondii (cartoon). The rough ER is connected to the basolateral margins of the outer nuclear membrane. Vesicles appear to bud away from the apical margin of the nucleus as small coated vesicles that enter the nearest Golgi stack. Clathrin-coated vesicles budding from the putative trans-Golgi contain material of electron density similar to that of the rhoptries. The net effect suggests a vectorial secretory pathway, progressing from the basal to the apical end of the parasite, and employing the nuclear envelope as an obligatory intermediate between ER and Golgi. (C) Detail of the apical end of a Toxoplasma nucleus (N). Vesicles containing a thin coat bud from (or fuse with) the outer nuclear envelope adjacent to the Golgi (arrowhead). Vesicles on the opposite side of the Golgi appear to be coated with clathrin (C). Bar, 0.1 μm.
transport from the ER to Golgi, and onward to secretory organelles at the anterior end of this highly polarized parasite. The ER is connected to the nuclear envelope in all eukaryotes (Palade, 1975), but use of the nuclear envelope as an obligatory intermediate compartment between the ER and Golgi in Toxoplasma would stand in contrast to mammalian cells, where transitional ER elements are dispersed throughout the cell (Bannykh et al., 1998; Saraste and Svensson, 1991). Budding from the nuclear envelope has also been observed in yeast (Bednarek et al., 1995).

**Localization of coatamer**
In yeast and mammalian cells, vesicles in transit between the ER and Golgi complex are covered with a thin proteinaceous coat formed by a complex of proteins comprising COP-I or COP-II (Bednarek et al., 1995; Hobman et al., 1998; Hong, 1998). Because the morphology of this coat resembles the vesicles seen budding from the nuclear envelope in Toxoplasma (see Fig. 1C), we tested whether this region is recognized by antiserum raised against recombinant T. gondii COP-I and COP-II proteins. cDNA clones exhibiting unequivocal similarity to mammalian β-COP (Duden et al., 1991; Serafini et al, 1991) and Sec23p (Barlowe et al., 1994; Paccaud et al., 1996) were identified in the T. gondii EST database (Ajioka et al., 1998), and expressed as recombinant fusion proteins for antibody production in rabbits (see Materials and methods). Immunofluorescence microscopy using anti-TgSec23p showed diffuse cytoplasmic staining (not shown), as has also been reported in mammalian cells and yeast, where most Sec23p is cytosolic (Hong, 1998). In contrast, anti-Tgβ-COP showed intense staining on the anterior surface of the nucleus (Fig. 2), and treatment with BFA results in dispersion of β-COP staining throughout the cell (not shown). Parasites just beginning the process of division (endodyogeny) exhibit a particularly revealing pattern of antibody staining: β-COP fluorescence divides into two distinct regions near the nuclear apex (arrowheads in Fig. 2), reflecting duplication of the Golgi during early division (Chobotar and Scholtyseck, 1982). Similar results were obtained using an antiserum that recognizes mammalian β-COP (not shown), but neither antibody could be detected by immunoelectron microscopy.

**Secretory pathway inhibitors**
If the above hypothesis that the nuclear envelope functions as an intermediate compartment between the ER and Golgi apparatus is correct, one would predict that disrupting the secretory pathway should affect the morphology of the nuclear envelope. It is now well established that the fungal metabolite brefeldin A (BFA) interferes with secretory transport in many cell types by blocking forward traffic from the ER to Golgi, while leaving the Golgi-to-ER retrieval

*Fig. 2. Immunofluorescence localization of β-COP. Top panel, FITC; middle, DAPI; bottom, merged FITC + DAPI + phase-contrast images. Polyclonal antiserum raised against a recombinant fragment of T. gondii β-COP (see Materials and methods) recognizes the apical juxtanuclear region of T. gondii tachyzoites (top). Intracellular parasites orient with their apical ends pointing outward (Morrisette et al., 1994); the apical end can also be recognized by the staining of extranuclear DNA associated with the apicoplast (Köhler et al., 1997) (arrow in center panel). The Golgi is among the first structures to divide in parasites undergoing mitosis (arrowheads). Bar, 4 μm.*
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pathway relatively intact (Klausner et al., 1992; Lippincott-Schwartz et al., 1998). In mammalian cells, BFA causes rapid disassembly of the Golgi, redistribution of Golgi membranes into the ER, and a concomitant increase in the size of both the ER and intermediate compartments (Klausner et al., 1992; Saraste and Svensson, 1991). BFA blocks protein secretion in *Plasmodium* as well, despite the absence of a readily visible Golgi (Crary and Haldar, 1992). In *Toxoplasma*, the ER nuclear envelope Golgi model for protein transport predicts that disappearance of the Golgi complex upon BFA treatment should be associated with swelling of the nuclear envelope. Similar effects should also be observed with other inhibitors of protein secretion, such as drugs that interfere with protein folding within the ER (Braakman et al., 1991; Umebayashi et al., 1997) or disrupt the microtubule cytoskeleton (Lippincott-Schwartz et al., 1990; Turner and Tartakoff, 1989).

As shown in Fig. 3A, BFA treatment disrupts the Golgi stack, leaving only a residual population of vesicles (some of which remain attached to the outer membrane of the nuclear envelope). In parallel, the nuclear envelope balloons to fill the cell cytoplasm with a maze of membranous compartments. This effect was first observed 40 minutes after treatment, and became more pronounced with prolonged incubation. Close examination of serial sections (not shown) indicates that the convoluted network of membranes observed in these BFA-treated parasites results from sectioning across massively swollen and folded extensions of the nuclear envelope. Swelling extends into the ER, which appears to be fully interconnected with the nuclear envelope. In contrast to the extensive malformation of basal aspects of the nuclear envelope, the anterior face of the envelope is not greatly altered, and numerous vesicles with thin coats are seen budding from (or fusing with) its surface. Additional uncoated vesicles are present anterior to these, but the flattened cisternae found in untreated *T. gondii* (Fig. 1C) are notably absent.

Treatment of parasites with dithiothreitol (DTT), which disrupts ER-to-Golgi trafficking due to improper protein folding (Braakman et al., 1991), produces bloating of the ER similar to that observed with BFA (not shown). In mammalian cells, microtubule destabilizing drugs such as nocodazole cause fragmentation of the Golgi complex (Ho et al., 1989; Turner and Tartakoff, 1989). Although colchicine is inactive against most protists, dinitroaniline herbicides such as ethalfluralin are capable of depolymerizing *T. gondii* microtubules (Stokkermans et al., 1996); the effect of ethalfluralin treatment on *T. gondii* parasites is shown in Fig. 3B. In addition to the absence of spindle pole body microtubules, the Golgi apparatus is also lost. The nuclear envelope expands and folds dramatically, extending far into the cytoplasm – precisely as seen with BFA treatment. Membranous extensions proliferate from the entire basal surface of the nuclear envelope, but not from the apical domain where the Golgi complex was originally located. Thus disruption of the Golgi apparatus in *Toxoplasma* by several independent means leads to pronounced perturbation of the ER/nuclear envelope.

![Fig. 3. Effect of inhibitors on architecture of the *T. gondii* secretory pathway. (A) Thin section through a *Toxoplasma* parasite treated with 20 μg/ml brefeldin A for 4 hours. The apical end of the nuclear envelope is unperturbed, but elsewhere the outer membrane has pulled away from the inner membrane of the nuclear envelope, making numerous complicated excursions into the basal cytoplasm. (B) Treatment with 10 μM ethalfluralin results in bloating of the nuclear envelope/ER compartment comparable to that observed in BFA. Treatment with DTT yields a similar effect (not shown). Go, Golgi. Bar, 1 μm.](image-url)
The ER-retention signal of *T. gondii* BiP causes arrest of a secretory GFP reporter within the nuclear envelope/ER compartment

In order to further examine organization of the ER in *Toxoplasma*, we sought to determine the retention signal associated with BiP, an ER-resident chaperone protein involved in controlling protein folding and exit from the ER (Pelham, 1989). Several sequences exhibiting similarity to BiP proteins from other species were identified in the *T. gondii* EST database (Ajioka et al., 1998) and used to obtain full-length cDNA clones, as described in Materials and methods. The complete sequence of *T. gondii* BiP is available from GenBank, with accession #AF110397. This sequence is virtually identical to that of the related apicomplexan parasite *Eimeria tenella* (GenBank #Z66492), 71% identical to *Plasmodium* (#Q05866) and 70% identical to human (#M19645). As in *E. tenella* (Dunn et al., 1996), the predicted C-terminus of *T. gondii* BiP consists of the amino acid sequence HDEL, which is similar to the KDEL motif found in mammals (Monro and Pelham, 1987) and the SDEL sequence in *Plasmodium falciparum* (Kumar and Zheng, 1992). To assess the functional importance of this sequence in ER retention, and to facilitate further morphological characterization of the *T. gondii* secretory pathway, we modified a secretory pathway reporter to contain a C-terminal HDEL. We have previously shown that a recombinant fusion modified a secretory pathway reporter to contain a C-terminal HDEL. We have previously shown that a recombinant fusion between the major *T. gondii* surface antigen SAG1 and GFP is efficiently secreted from transgenic parasites (Striepen et al., 1998). Secretion into the parasitophorous vacuole reveals the tachyzoites as dark shadows (Fig. 4A). Addition of HDEL to this construct, however, causes the GFP signal to be retained intracellularly, as shown in Fig. 4C. Transgenic parasites expressing the GFP-P30-HDEL construct exhibit GFP fluorescence as a ring surrounding the nucleus. Staining is particularly intense in the cup-like region at the apical end of the nucleus, adjacent to the Golgi (arrowhead). Apical concentration of GFP fluorescence is particularly dramatic in dividing parasites (not shown).

![Fig. 4.](image)

**DISCUSSION**

**The vectorial secretory pathway of *Toxoplasma gondii***

Several lines of evidence support the view that the streamlined secretory apparatus of *Toxoplasma* employs the nuclear envelope as an intermediate compartment between the ER and the Golgi complex. (1) Morphological studies indicate that the ER is predominantly basal, while the Golgi and specialized secretory organelles are restricted to the parasite’s apical end. The large central nucleus poses a significant impediment to vesicular basal-to-apical transit (Fig. 1A). (2) The Golgi is closely apposed the nuclear envelope throughout the entire cell cycle (the nuclear envelope does not break down during mitosis in *T. gondii*), and numerous vesicles appear to traffic between the apical surface of the nuclear envelope and the Golgi (Fig. 1C). Vesicles between the apical end of the nucleus and the Golgi exhibit a thin coat comparable to coatomer in mammalian cells (Fig. 1C), and anti-β-COP stains this region intensely (Fig. 2). (3) Pharmacological treatments that disrupt ER-to-Golgi traffic in mammalian cells cause bloating of the nuclear envelope in *T. gondii* (Fig. 3). This morphology is strikingly reminiscent of mutations in the yeast secretory pathway, where a block at one step results in dilation of the previous compartment (Novick et al., 1981; Rothman and Orci, 1992; Umebayashi et al., 1997). (4) Addition of an ‘HDEL’ ER-retention motif to the C terminus of secretory reporters causes these proteins to be retained in the nuclear envelope (Fig. 4). The outer nuclear membrane of many eukaryotes is studded with ribosomes, and presumably functions as an adjunct to the ER in secretory protein synthesis (Palade, 1975); in yeast,
COP-coated vesicles have been observed budding from the ER (Bednarek et al., 1995). What is unusual about Toxoplasma is that the nuclear envelope appears to function as an intermediate compartment in a polarized protein secretory pathway, connected physically with the ER, and via vesicles to the Golgi apparatus. In this model, secretion follows a defined vectorial pathway, proceeding from ER to nuclear membrane to Golgi, and on to the specialized secretory organelles at the parasite’s apical end (Carruthers and Sibley, 1997; Dubremetz, 1998).

These studies suggest that the apical surface of the nuclear envelope is functionally distinct from the basal domain in Toxoplasma. Pre-Golgi coated vesicles emerge only from this region, and the ER is never connected here. What makes the apical region unique remains unclear, but it may prove homologous to transitional elements of the mammalian secretory pathway (Bannykh et al., 1998; Saraste and Svensson, 1991).

**Intracellular protozoan parasites as stripped-down eukaryotic models**

Intracellular parasitic protozoa, by virtue of their small size, provide stripped-down examples of eukaryotic design, which may allow us to appreciate the minimal equipment necessary for secretion. As minute organisms branching off the eukaryotic lineage prior to the divergence of animals, fungi and plants (Levine, 1988; Sogin, 1991), it is likely that studies on these organisms will also yield new insights into evolution of the eukaryotic secretory pathway (Becker and Melkonian, 1996).

Among apicomplexan parasites, Toxoplasma is perhaps the most convenient organism for study, because of its experimental accessibility (Boothroyd et al., 1994; Roos et al., 1994). The ability to visualize and reconstruct the entire secretory apparatus of a cell at high resolution – particularly in a highly polarized protist where the net direction of secretory protein transport is known (Dubremetz, 1998) – may provide the means to test theories regarding the mechanisms of transport thorough the Golgi apparatus and functional microdomains within secretory pathway organelles (Mellman and Simons, 1992; Rothman and Orci, 1992; Nickel et al., 1998; Verkade and Simons, 1997). Such research should complement studies on yeast (Bednarek et al., 1995; Schekman, 1992; Umebayashi et al., 1997) by providing an organism that is easy to interpret in morphological terms, and for which genetic experiments are also feasible (if somewhat less convenient than in S. cerevisiae). Taking advantage of secretory molecules such as the P30-GFP-HDEL reporter (Fig. 4), it should be possible to develop screens for conditional secretory pathway mutants (Striepen et al., 1998).

Toxoplasma also provides a convenient bridge for understanding the architecture of even more highly simplified organisms. In this context, it is interesting to speculate on why T. gondii possesses such a striking Golgi apparatus, in contrast to parasites such as Plasmodium, where the Golgi is difficult to recognize morphologically (Crary and Haldar, 1992; Ward et al., 1997). Protein glycosylation is less common in Toxoplasma (Dieckmann-Schuppert et al., 1994; Kimura et al., 1996), but both parasites require extensive protein targeting, particularly during assembly of the apical secretory organelles (Carruthers and Sibley, 1997; Striepen et al., 1998). Unlike Toxoplasma, which replicates its DNA and undergoes cytokinesis approximately every 7 hours (Fichera et al., 1995), however, Plasmodium and Theileria form a polyploid nucleus via multiple cycles of DNA replication, followed by multiple rounds of cytokinesis after 48 hours (a process termed schizogony; Shaw and Tilney, 1992).

Preliminary studies indicate that coated vesicles which may represent the Plasmodium Golgi are recognizable during schizogony (when the rhoptries and micronemes are being formed); such stage-specific regulation of secretory organelles provides an intriguing mechanism for conserving space in the highly constrained environment of an intracellular parasite.

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