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Identification of endoplasmic reticulum in the primitive eukaryote *Giardia lamblia* using cryoelectron microscopy and antibody to BiP

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SUMMARY

***Giardia lamblia* trophozoites contain a complex endomembrane system as demonstrated by fluorescence and cryoelectron microscopy. The endomembrane system was weakly detected in live cells using the fluorescent membrane dye 3,3′-dihexyloxacarbocyanine iodide. The definitive identification of endoplasmic reticulum required the development of a molecular label. We expressed Giardial Bip in *Escherichia coli* and raised a polyclonal antibody to the purified protein. In western blots, the antibody was specific for Giardial Bip and did not react with human, monkey and rodent homologs. By immunofluorescence microscopy in methanol fixed cells the antibody visualized tubular structures and other subcellular components that required characterization by electron microscopy. Using cryotechniques we directly demonstrate the presence of a complex endomembrane system at the ultrastructural level. In conjunction with Bip immunogold labeling of cryosections we identify: (1) endoplasmic reticulum cisternae and tubules; (2) stacked perinuclear membranes; and (3) Bip presence in the nuclear envelope. Both the endoplasmic reticulum and nuclear envelope were found either with or without a cleft region suggesting each may contain common specialized sub-regions. In stacked perinuclear membranes, which may represent either multilamellar endoplasmic reticulum or a Golgi apparatus, Bip labeling was restricted to peripheral layers, also suggesting specialized sub-regions. Labeled endomembrane systems could be observed associated with microtubule structures, including axonemes and the adhesive disk. The presence of an extensive endomembrane system in *Giardia lamblia*, which represents one of the earliest diverging eukaryotic species, supports the view that both the nucleus and endomembrane system co-evolved in a common ancestor of eukaryotic cells.**

Key words: Bip, Endoplasmic reticulum, Golgi apparatus, Giardia

INTRODUCTION

Giardia lamblia represents one of the deepest branching or most primitive eukaryotes in existence (Sogin et al., 1989; Gupta et al., 1994), and is further distinguished because it lacks many of the subcellular organelles characteristic of higher eukaryotes, including mitochondria and peroxisomes (Friend, 1966; Feely et al., 1990; Adam, 1991). As such, this flagellated protozoan may provide our closest glimpse into the origins of the eukaryotic cell. The endoplasmic reticulum (ER) was first discovered and characterized by Keith Porter in mammalian cells (e.g. Porter, 1953). To this date our appreciation of ER structure and distribution has been derived from studies primarily on mammalian cells and there is a paucity of information on endomembranes in primitive eukaryotes. Although the fine structure of Giardia was first described 30 years ago (Friend, 1966), the very existence of ER has been doubted, even recently (Feely et al., 1990; Meyer, 1994). On the one hand, a lack of ER would appear to violate a central dogma in the evolution of the eukaryotic cell, namely that the endomembrane system and nucleus co-evolved in the same evolutionary event and that all eukaryotic cells would possess both (Alberts et al., 1994). On the other hand, if ER is present it may be present in its simplest evolutionary form and thus the study of Giardia may provide fundamental insights into intracellular organization.

This laboratory recently obtained the first molecular evidence that ER must be present in Giardia after isolating the gene for Bip (Gupta et al., 1994). Bip is a hsp70 homolog which resides in the lumen of the ER in higher eukaryotes. In higher eukaryotes, including yeast, Bip functions as a molecular chaperone in protein folding and in the translocation of proteins across the ER membrane (Craig et al., 1993). Giardial Bip was found to contain a classic C-terminal KDEL ER retention signal (Gupta et al., 1994), implying that ER membranes would be present in this organism. Since the KDEL signal acts in the retrieval of ER proteins that have entered the Golgi (Alberts et al., 1994), then the existence of a Golgi complex is also probable. We have now raised an antibody that is specific for Giardial BiP and have localized the protein at both the light and electron microscopic levels. The definitive identification of ER, however, also required demonstrating the presence of endomembranes in Giardia. The endomembranes of Giardia have eluded most past investigators. Most ultrastructural studies to date have dealt primarily with the organization of the cytoskeleton (see Adam, 1991; Soltys and Gupta, 1994a). Friend (1966) using conventional

EM methods originally reported that the only intracellular membranes that are present in Giardia are the nuclear envelope and the peripheral vacuoles that underlie the dorsal surface. The peripheral vacuoles are currently thought to be either secretory organelles (Friend, 1966; Reiner et al., 1990), endocytic vacuoles (Bockman et al., 1968; Tai et al., 1993) or to be lysosome-like (Feely and Dyer, 1987; Lindmark, 1988; McCaffery and Gillin, 1994). Structures with resemblance to ER, however, have been observed in electron micrographs (Reiner et al., 1990; McCaffery and Gillin, 1994) but were not identified with a definitive molecular marker.

To identify ER membranes in Giardia and to characterize their morphology and subcellular distribution, we have in this paper first of all adopted the cryotechniques of Tokuyasu (1986) to demonstrate the presence of an extensive endomembrane system in Giardia. The advantages of cryosections prepared by these procedures include enhanced membrane preservation and, since cells are not embedded in plastic nor osmicated to fix/contrast membranes, there is high epitope reactivity during antibody labeling. Second, to obtain an ER marker, we report the expression of recombinant Giardial BiP in *E. coli* and the development and characterization of an antibody to it. We have used this antibody in immunogold labeling of cryosections to provide the first definitive identification of ER in Giardia using a molecular marker that is specific for the ER network. We have also identified stacked perinuclear membranes in trophozoites that, based on morphology and partial labeling with BiP antibody, may represent the Golgi apparatus. An overview of the ER network at the light microscopic level was also obtained by visualizing endomembranes in living trophozoites with $DiOC_6(3)$ cyanine dye labeling (Terasaki et al., 1984; Soltys and Gupta, 1992), or by immunofluorescence labeling in fixed cells with Bip antibody. The results indicate Giardia has an unexpectedly extensive endomembrane system, the characteristics of which are described.

MATERIALS AND METHODS

Cell culture

G. lamblia WB (ATCC 30957) trophozoites were grown in glass culture tubes in TY1-S-33 medium supplemented with bile (Keister, 1983). Trophozoites growing as monolayers were harvested by washing and chilling in PBS (containing 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 and 0.085 g KH_2PO_4 per liter, at pH 7.2), followed by centrifugation. Human fibroblasts, B-SC-1 African green monkey kidney cells and Chinese hamster ovary cells were grown according to previously described procedures (Soltys and Gupta, 1992). All cells were free of mycoplasma contamination.

Bacterial expression of Giardial Bip

Based on the nucleotide sequence of Giardial Bip, forward and reverse oligonucleotide primers which flanked the coding sequence for the protein and containing unique restriction enzyme sites (*Eco*RI and *Hin*dIII, respectively) were custom synthesized (Mobix - The Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton, ON). The nucleotide sequence of the primers employed were as follows: (forward) 5′-GAATTCCATGCTCGCTCTTGTCTTTGCC-3′ and (reverse) 5′-GGATCCAAGCTTAGAGTTCATCTTTTTCTGC-3′. These primers were employed in polymerase chain reaction (PCR) in conjunction with DNA from the plasmid HSG-12, which contained the cloned Giardial Bip gene (Gupta et al., 1994). After 35 PCR cycles, specific amplification of a 1.9 kb fragment was observed, as expected based on the positions of the primers. The fragment was isolated and after digestion with *Eco*RI and *Hin*dIII, it was subcloned in the pTrcHis expression plasmid, digested with the same enzymes. The sequencing of the plasmid (following transformation of *E. coli* JM109 cells) confirmed that the insert was in the correct reading frame. The DNA from this plasmid (designated pGLBip-1) was employed to transform *E. coli* Top10 cells for expression of the recombinant protein. However, no significant expression of the recombinant protein was observed using this construct.

The construct used in the above experiment contained the entire coding sequence of the Giardial Bip, including the N-terminal ER targeting presequence, which is not present in the mature protein. Since this sequence could adversely affect the expression, or even may be causing secretion of the recombinant protein, the plasmid pGLBip-1 was digested with the enzyme *Ava*I and then religated. This led to removal of a portion of the 5′-end sequence corresponding to the N-terminal 47 amino acids. The resulting clone (designated pGLBip-2) contained 615 amino acids of the Giardial Bip sequence and it showed very good expression of the recombinant protein.

Expression and purification of recombinant protein

An overnight culture of *E. coli* cells harbouring pGLBip-2 plasmid was diluted 1:100 in fresh growth medium containing 100 μg/ml ampicillin and grown at 37°C. After 1-2 hours when the OD_{660} of the culture was between 0.4-0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 0.5 mM. No IPTG was added to a parallel control culture. At various times afterwards, samples of induced and uninduced cultures were taken out and expression of the recombinant protein was examined by SDS-PAGE. For large scale preparation of the recombinant protein, the culture was induced with IPTG for 3 hours and the washed cell pellet was resuspended in 20 volumes of lysis buffer (6 M guanidine hydrochloride, 20 mM sodium phosphate and 500 mM sodium chloride, pH 7.8). After clearing the lysate by centrifugation (5,000 rpm for 15 minutes), the supernatant was applied to a prewashed Probond Nickel column (Invitrogen Corp., San Diego, CA). The recombinant protein which contains a poly-His_6 tag binds to the Ni column. After washing the column with buffers of decreasing pH (as per the supplier's protocol), the bound protein was eluted using a buffer containing 8 M urea, 20 mM sodium phosphate, 500 mM NaCl, pH 4.0, and desalted. SDS-PAGE analysis of the eluted protein showed that it consists mostly (>90%) of a ≃70 kDa protein and a few other minor protein bands of lower molecular masses, which presumably were proteolytic degradation products. Nevertheless, for antibody production, the ≃70 kDa protein band was excised from preparative SDS-PAGE gels and the eluted protein was lyophilized and used as antigen.

Polyclonal antibody to the recombinant Bip was raised by injecting about 1 mg of the recombinant protein emulsified with 1 ml of Titre Max (Cedarlane Laboratories, Hornby, ON) subcutaneously in a female rabbit. After 4-5 weeks, when the animal showed an immune response against the injected antigen, a second booster dose of the antigen in Titre Max was given. The animal was bled within the next 2-3 weeks. In most of the experiments described the polyclonal antibody was further affinity purified using the recombinant protein.

Gel electrophoresis and western blots

Giardia cellular extracts for polyacrylamide gel electrophoresis and western blots were prepared by boiling a pellet of PBS-washed cells for 10-15 minutes in Laemmli sample buffer containing 2% SDS, 100 mM dithiothreitol, 60 mM Tris-HCl, pH 6.8, and 0.001% bromophenol blue. To prevent proteolysis in sample preparation, ice-cold sample buffer was added to the pellet on ice, the pellet was resuspended by repeated pipetting, then the tube was transferred directly from ice to a boiling water bath. For tissue culture cells, Laemmli sample buffer was added to PBS-washed monolayer cultures and boiling was for 3 minutes. Electrophoresis was in 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE), as described previously (Gupta and

Dudani, 1987). Proteins were transferred electrophoretically from polyacrylamide gels to nitrocellulose sheets. The blots were blocked with 3% bovine serum albumin (BSA) in saline (0.9% NaCl, 10 mM Tris-HCl, pH 7.4), then reacted with affinity purified rabbit polyclonal antibody to Bip. Visualization of polyclonal antibody was with horseradish peroxidase conjugated secondary antibody directed against rabbit IgG (Bio-Rad Lab. Ltd, Mississauga, ON) and color development with 4-chloro-1-naphthol (Bio-Rad Lab. Ltd, Mississauga, ON).

Immunocytochemistry and microscopy

For light microscopy a concentrated suspension of trophozoites in ice-cold TY1-S-33 culture medium was applied to ethanol-prewashed glass coverslips and trophozoites were allowed to adhere for 5-10 minutes at 37°C in a humidified incubator. For cyanine dye staining of living cells, $DiOC_6(3)$ (3,3′dihexyloxacarbocyanine iodide) (Sigma, St Louis, MO) was used at 5 μg/ml in Giardia culture medium for 40 seconds, as described previously for mammalian cells (Soltys and Gupta, 1992). For immunofluorescence labeling, trophozoites on coverslips were fixed by quickly plunging into −20°C methanol. After fixation for 15 minutes at −20°C, cells were rinsed with PBS, then antibody labeled. Immunofluorescence procedures and microscopy were otherwise described previously (Soltys and Gupta, 1992).

For electron microscopy, PBS washed cells on ice were fixed as a loose pellet of cells with 0.5% glutaraldehyde in 0.1 M sucrose, 0.1 M cacodylate, pH 7.3, at room temperature for 15 minutes (Soltys and Gupta, 1994a,b). To quench unreacted glutaraldehyde, cells were washed and incubated for 15 minutes in 100 mM ammonium chloride in sucrose-cacodylate buffer. Pellets of cells were infiltrated with 2.3 M sucrose for 3 hours (Soltys and Gupta, 1996). The general cryomicrotomy procedures of Tokuyasu (1986) were used. Ultrathin cryosections were cut on a Reichert-Jung ultra cut E ultramicrotome with the FC 4E cryosectioning attachment (knife −85°C; specimen −90°C; chamber −110°C). Antibody labeling of cryosections when using 10 nm gold labels was carried out using a three-stage immunolabeling procedure to amplify labeling intensity (Tokuyasu, 1983; Soltys and Gupta, 1994b, 1996). Sections were preabsorbed at room temperature with 50% fetal calf serum in 0.1 M Tris-HCl, pH 7.5 (carrier buffer). Sections were then reacted with affinity-purified polyclonal antibody in carrier buffer for 1.5 hours at 37°C in a humidified incubator. Washing of sections was for 30 minutes with 5% BSA in 0.1 M Tris-HCl, pH 7.5. Sections were then reacted with a 1:40 dilution of goat anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA) in carrier buffer for 1 hour at 37°C, washed again, then reacted with a 1:5 dilution (A_{520}=0.5) of rabbit anti-goat IgG 10 nanometer gold conjugate (Sigma Chemical Co., St Louis, MO) in carrier buffer for 4 hours at 37°C. When 18 nm gold particles were used, a two-stage procedure was used and the second antibody was a goat anti-rabbit IgG 18 nm gold conjugate (BioCan Sci., Mississauga, ON). After washing, including a high salt wash with 0.5 M KCl in carrier buffer followed by washes with H_2O, cryosections were stained with 2% neutral uranyl acetate, then embedded in methylcellulose containing 0.1% acidic uranyl acetate. Sections were examined at 80 kV with a JEOL 1200 EX transmission electron microscope.

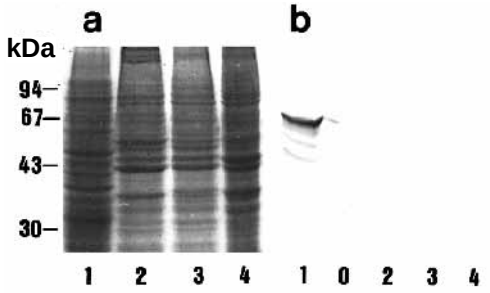

Fig. 1. Immunoblot detection of Bip in *G. lamblia* trophozoites. (a) SDS-PAGE. Coomassie blue staining of the gel. (b) Western blot analysis of trophozoites (lane 1) compared with human fibroblasts, B-SC-1 monkey kidney cells and Chinese hamster ovary cells (lanes 2-4), respectively. Lane 0 in b is a blank lane. Immunoblot in b was stained with affinity purified polyclonal antibody to Giardial Bip and a secondary horseradish peroxidase-conjugated antibody.

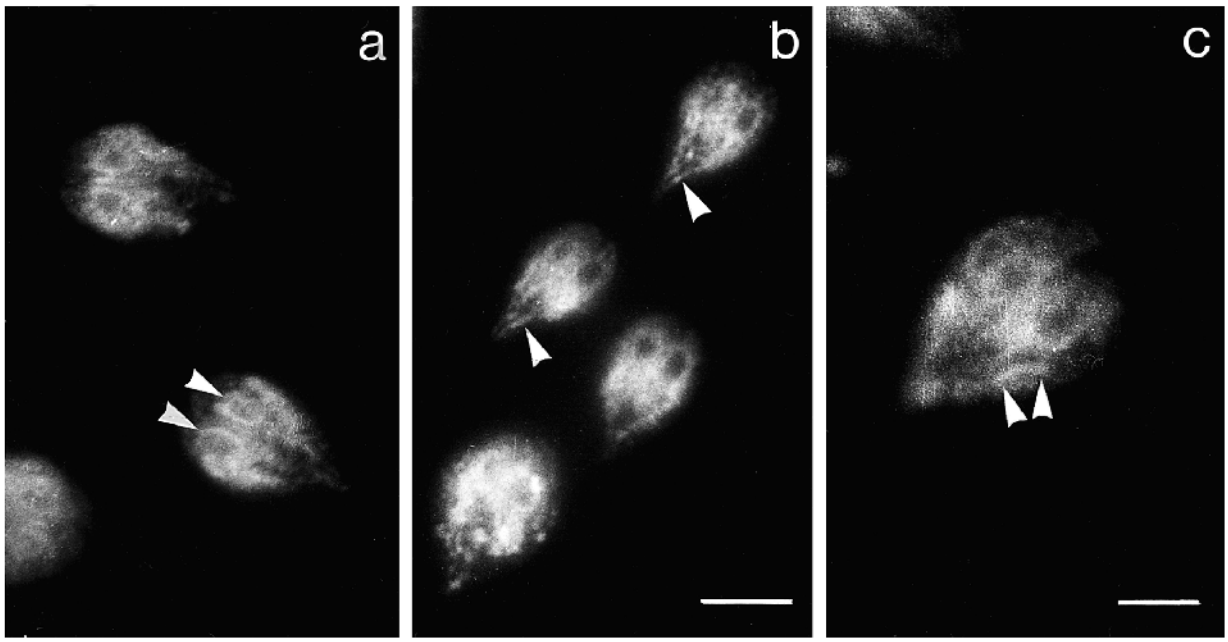

Fig. 2. Fluorescence visualization of endomembranes in *G. lamblia* trophozoites. (a) Cyanine dye $DiOC_6(3)$ staining in living cells. Fluorescein channel. (b and c) Bip antibody labeling in fixed cells. Fluorescein channel. Staining of the nuclear envelope is most prominent in a (see arrowheads). Cytoplasmic tubular structures are prominent in b and c (see arrowheads). The indicated tubular structure in c appears to be greater than 3 μm in length. Bars: (b) 10 μm; (c) 5 μm.

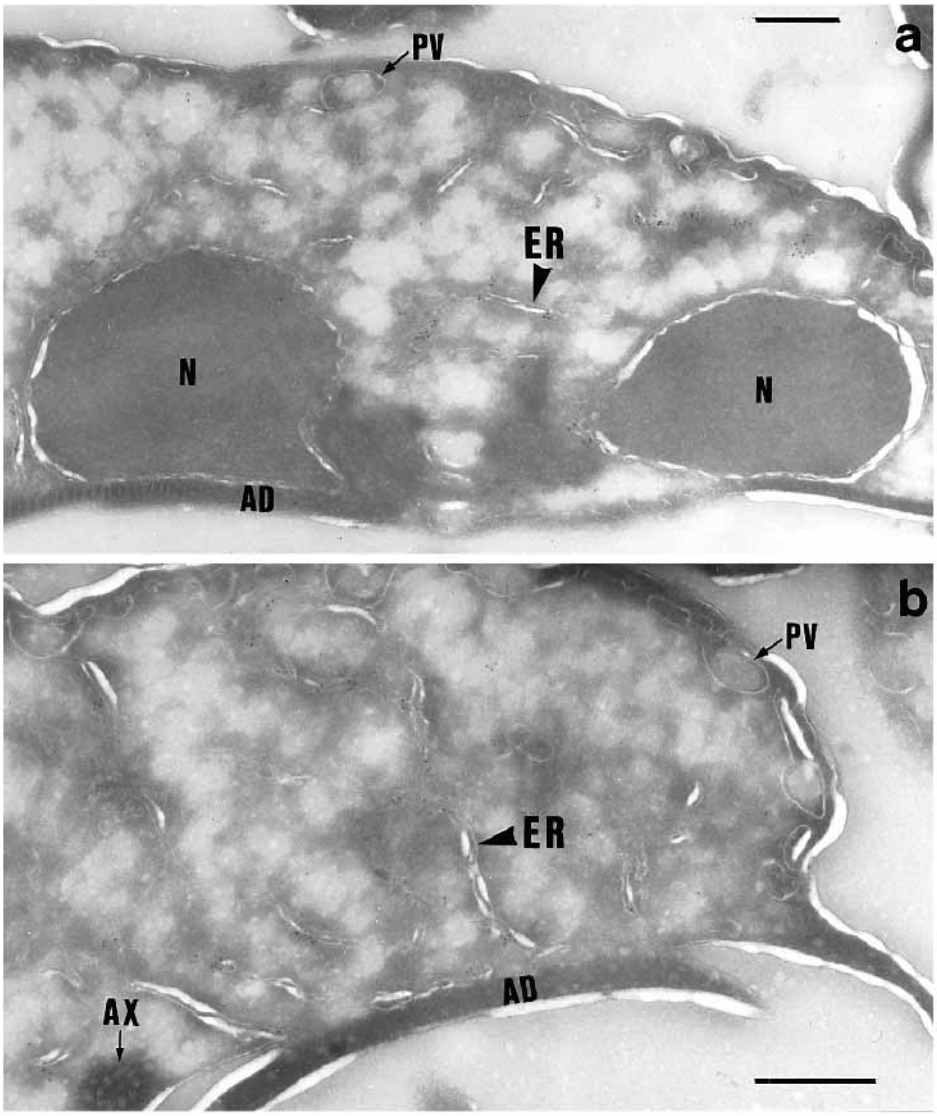

Fig. 3. Cryoelectron microscopy of *G. lamblia* trophozoites and immunogold labeling of endomembranes with antibody to Giardial Bip. Low magnification overviews; 10 nm gold conjugates used, which would be observed with a low power magnifier. ER, endoplasmic reticulum as identified by Bip labeling; AD, adhesive disk, AX axoneme, PV, peripheral vacuole; N, nucleus. Bars, 0.5 μm.

RESULTS

Previous morphological studies have shown that Giardia has a very primitive cytoplasm that lacks organelles such as mitochondria and peroxisomes. We asked whether ER is present and how does it compare morphologically with ER in higher eukaryotes. The definitive identification of any endomembrane components as being ER requires a molecular label, so we produced an antibody to the ER resident molecular chaperone Bip. The Bip gene from *G. lamblia* has been cloned and sequenced in our laboratory (Gupta et al., 1994). In the present study we expressed histidine tagged recombinant Bip protein in *E. coli*, purified it on a nickel column, then raised a rabbit polyclonal antibody to the purified protein (see Materials and Methods for details).

The specificity of the Bip antibody was evaluated in *G. lamblia* whole cell extracts and compared with other species. Fig. 1a and b are polyacrylamide gels and immunoblots, respectively, of *G. lamblia* (lane 1) compared with human fibroblasts, B-SC-1 monkey kidney cells and Chinese hamster ovary cells (lanes 2-4, respectively). A 70 kDa protein was strongly labeled in Giardia. No protein bands were labeled in the other cell types. In Giardia, faint lower molecular mass bands were also detected, probably representing minor proteolysis. Thus, the Bip antibody is species specific and does not react with rodent or primate cells.

We first evaluated endomembranes in live cells using fluorescence dye labeling. The cyanine dye $DiOC_6(3)$ has previously been used to visualize ER and mitochondria in live or fixed mammalian cells (Terasaki et al., 1984; Soltys and Gupta, 1992). In the absence of mitochondria in Giardia this dye might be expected to visualize primarily ER. Fig. 2a shows $DiOC_6(3)$ staining of live trophozoites. There is strong fluorescence staining of the nuclear envelope/periphery (arrowheads) and there are other fluorescent structures distributed throughout the cytoplasm. These structures appear to have both linear and punctate shapes. $DiOC_6(3)$ therefore is likely labeling endomembranes and these are presumably ER. The signal intensity is low, however, and the shape and distribution of these subcellular components is less amenable to characterization than ER in mammalian cells (Terasaki et al., 1984; Soltys and Gupta, 1992). It should be noted, however, that these were living cells and there were significant subcellular movements and photobleaching of fluorescence during photography, causing image loss. Fig. 2b and c in comparison show immuno-

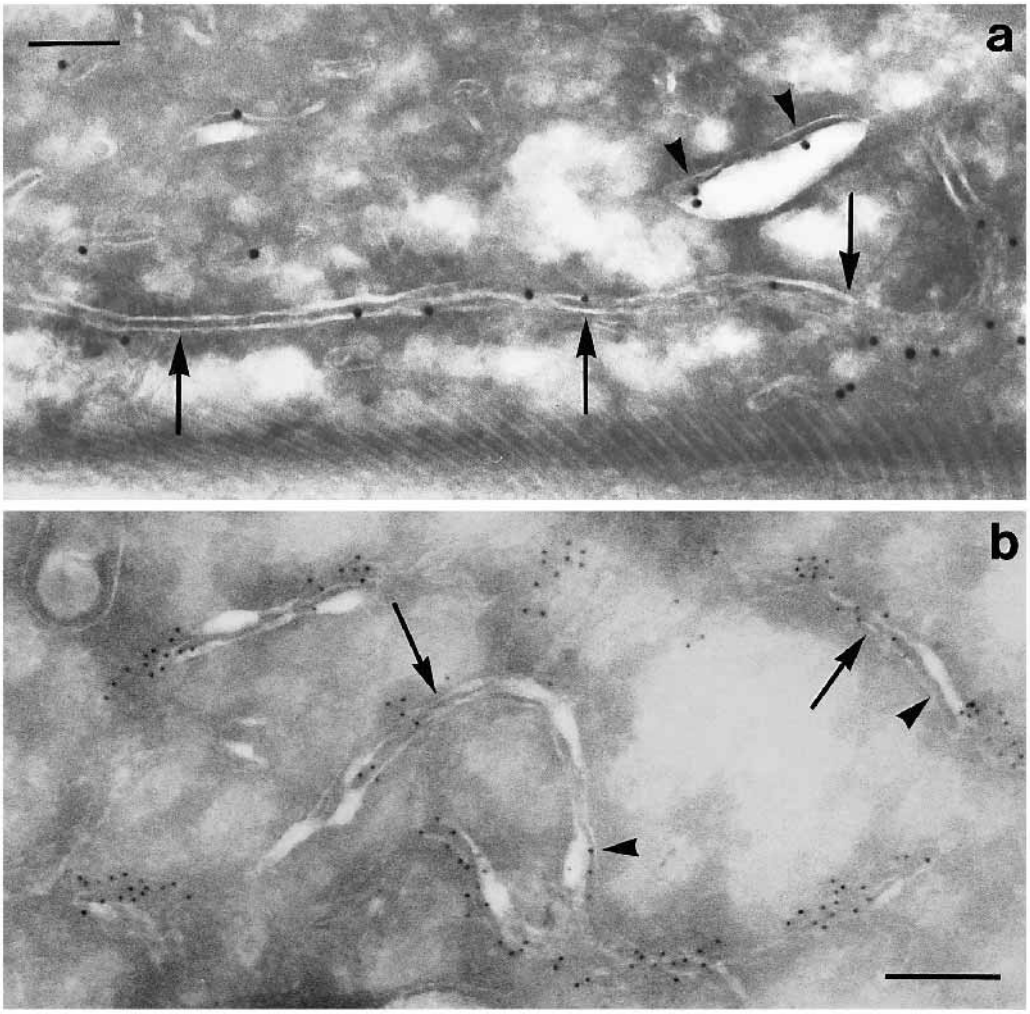

Fig. 4. Immunogold labeling of *G. lamblia* cryosections with Bip antibody. High magnification micrographs of ER membranes: (a) 18 nm gold labels; (b) 10 nm gold labels. In a, arrows denote a single ER tubule which is approximately 2 μm in length. A cytoplasmic cleft is also present in which a limiting membrane is detected along its upper border and is also gold labeled (arrowheads). In b, labeling of ER membranes is increased when 10 nm gold particle procedures are used. ER membranes with cytoplasmic clefts (arrowheads) and without (arrows) are present and both are labeled. These ER regions appear continuous with each other. Bars, 0.2 μm.

fluorescence labeling of fixed cells using Bip antibody. A fluorescent array throughout the cytoplasm is also detected by this approach, showing both tubular (arrowheads) and punctate or vesicle-like characteristics (Fig. 2b, cell in lower left corner), and there appears to be less labeling of the nuclear envelope compared with Fig. 2a. Control cells reacted with preimmune serum showed no labeling (not presented). The cell shown in Fig. 2c is at higher magnification compared with Fig. 2b. The indicated tubular structure, assuming it is a singular entity, is more than 3 μm in length. Thus both dye staining and fluorescent antibody labeling detect similar subcellular components that are present throughout the cytoplasm. While these results suggest the presence of an extensive system of endomembranes, the identification and characterization of ER membranes must be made at the ultrastructural level.

While previous morphological studies had difficulty preserving Giardia endomembranes, we were able to detect ER-like membranes by applying cryotechniques. Fig. 3a and b show low magnification overviews of two different regions of the cell. Fig. 3a is a central section through the two nuclei while Fig. 3b is a peripheral region of the cell. A prominent endomembrane component in both sections is the previously described peripheral vacuoles that underlie the dorsal surface of the cell. The peripheral vacuoles are thought to be secretory organelles (Friend, 1966; Reiner et al., 1990), endocytic vacuoles (Bockman et al., 1968; Tai et al., 1993) or to be lysosome-like (Feely and Dyer, 1987; Lindmark, 1988; McCaffery and Gillin, 1994). In addition, cytoplasmic clefts (labeled ER) are present that are apparently devoid of contents. These clefts were first described by Friend (1966) who did not observe them to contain membranes. These cytoplasmic clefts are abundant and present throughout the cytoplasm. Contrary to previous reports, we found in cryosections that these cytoplasmic clefts are membrane limited (see below). In addition we have also observed equally abundant ER-like membranes that do not contain an empty cleft. In addition to these membranous structures, we have also observed numerous vesicles throughout the cytoplasm that may be transport vesicles or cross-sections of ER tubules, and also novel stacked multilamellar membranes (see below).

We labeled cryosections with antibody to Giardial Bip to identify ER. Fig. 4a and b show high magnification micrographs of membranes labeled with 20 nm and 10 nm gold labels, respectively. In Fig. 4a, a single ER-like tubule can be tracked along its length for a distance of approximately 2 μm and it is labeled along the entire observed length. A cytoplasmic cleft is also present (top right quadrant) in which a membrane is clearly identified on its top edge and it is also antibody labeled. Therefore both these membranous structures are ER membranes. Gold labeling intensity of these membranes is

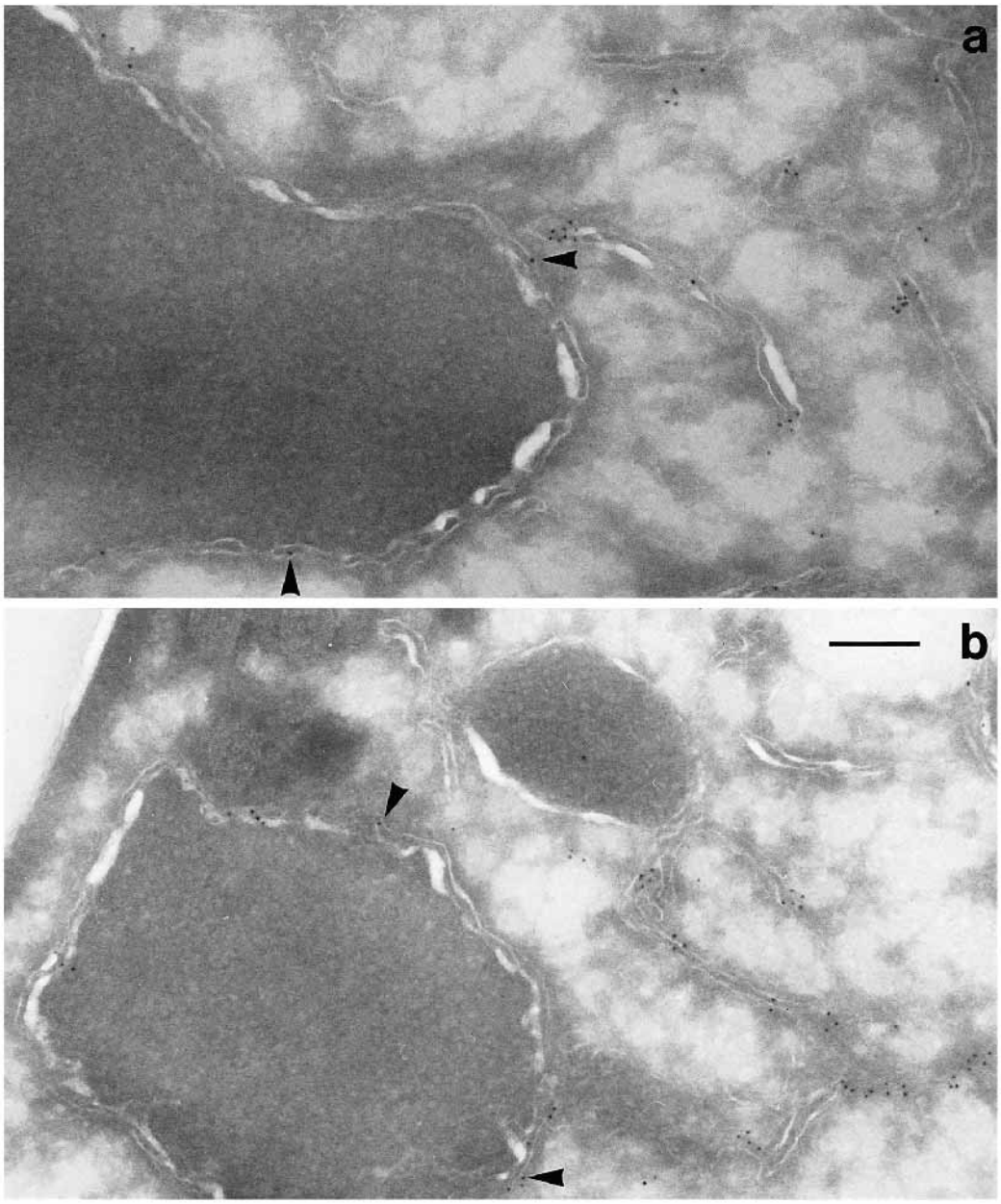

Fig. 5. Bip immunogold labeling of the nuclear envelope and juxtanuclear ER tubules. Some of the sites of the nuclear envelope that are labeled are indicated with arrowheads. Nuclear pores are observed and the nuclear envelope has several cleft regions. The nuclear envelope and the adjacent ER are morphologically similar. Note that the ER tubule in a immediately to the right of the nucleus has both cleft and non-cleft regions. 10 nm gold labels. Bar, 0.25 μm.

increased using our 10 nm gold labeling procedures as shown in Fig. 4b. Labeling again is demonstrated to be on membranes bordering cytoplasmic clefts and in the more typical ER-like membranes lacking a cleft. This is therefore proof that Giardia does indeed have ER. Control sections reacted with preimmune serum showed no labeling (not presented). We have not detected any difference in antibody labeling between the cytoplasmic clefts and the more typical ER membranes. The cleft itself may arise from a component that is readily extracted, so a compositional difference between these sets of membranes is likely. However, cytoplasmic clefts were often found to be continuous with non-cleft regions (Fig. 4b). The low magnification cryosections presented in the preceding Fig. 3 were in fact also antibody labeled (10 nm gold) and an overview of the distribution of gold particles on membranes throughout the cell can be obtained by use of a low power magnifier.

We have also obtained Bip labeling of the nuclear envelope. Fig. 5a and b show two nuclear regions at high magnification. Ten nanometer gold particles were used. ER tubules in the cytoplasm in close proximity to the nuclei are labeled. The nuclear envelopes are also labeled at several locations as indicated by arrowheads. This nuclear labeling is consistent with the view that ER membranes form the nuclear envelope and that Bip has functions at this location also.

We have observed novel multi-layered stacks of membranes in trophozoites, usually in a juxtanuclear location, and these have also been found to contain Bip. Fig. 6a and b show two examples of such stacks labeled with 10 nm gold particles. The stacks extend over a large area, often up to 1-2 μm in width. The intermembrane spacing in these stacks is approximately twofold less than in the general cytoplasmic ER. Note that Bip labeling is restricted to particular layers, suggesting functional specializations in different layers. The structural, positional and compositional properties of these stacked membranes suggest

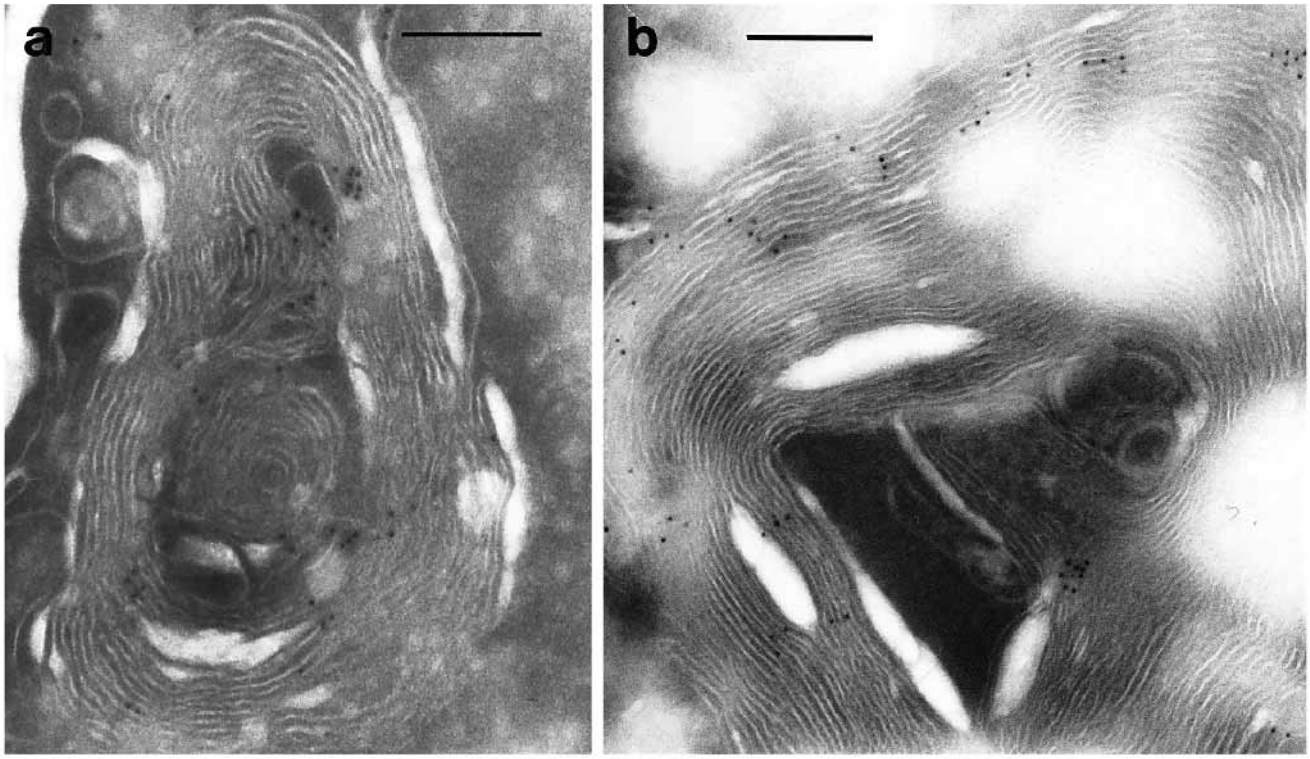

Fig. 6. Bip immunogold labeling of stacked membrane systems in vegetatively growing trophozoites. Bip labeling is restricted to specific layers, suggesting specialized subregions. The membrane-to-membrane spacing of these stacked cisternae is compressed relative to ER tubules or the nuclear envelope. 10 nm gold labels. Bars, 0.2 μm.

they are different from ER and could possibly represent a primitive Golgi apparatus (see Discussion).

We have found that endomembranes in Giardia are also associated with microtubule structures including the adhesive disk and axonemes. Fig. 7a shows stacked membranes labeled with Bip antibody in association with microtubules of the adhesive disk. Microtubules in the adhesive disk are viewed in cross section (arrowheads), which are at the bottom of so called 'microribbons' composed of the protein giardin (see Adam, 1991). In Fig. 7b, ER tubules (arrows) are found in close association with an intracytoplasmic axoneme. Associations of endomembranes with microtubule-based structures is a hallmark of higher eukaryotes.

DISCUSSION

To develop a molecular marker that can identify ER in *G. lamblia*, we expressed Giardial BiP in *E. coli* and raised an antibody to the purified protein. The specificity of this antibody was established by western blots and by the specific labeling of endomembranes by immunocytochemical procedures. This antibody was found to be species specific and does not react with rodent or primate cells.

By immunofluorescence microscopy using anti-Bip antibody, we obtained an overview of the distribution of ER membranes in whole cells. The ER network was found to be extensive and present throughout the cell. This network shares with higher eukaryotes tubular membrane profiles and there were also nondescript patches of membranes that were labeled. These components were also visualized in living trophozoites using the cyanine dye $DiOC_6(3)$. With either approach, however, the ER network had low signal intensity and was not as clearly defined as in previous fluorescence microscopy of mammalian cells (Terasaki et al., 1984; Soltys and Gupta, 1992). This finding of an extensive ER network in Giardia may not have been expected because of the primitive evolutionary position of this species (Sogin et al., 1989; Gupta et al., 1994).

By applying cryotechniques we were able to preserve giardial endomembranes at the ultrastructural level. This permitted us to identify the ER by immunogold labeling. Cisternae and tubular forms of ER were identified and were found throughout the cell body, often associated with other structural elements including the nucleus, peripheral vacuoles and complex microtubule-based structures such as intracytoplasmic axonemes and the adhesive disk. Labeled vesicles were also observed that may represent transport vesicles or cross sections of ER-tubules. The tubular ER could be distinguished into two subregions, namely those with or without a cleft region. Friend (1966) also observed cytoplasmic clefts and, although limiting membranes were not preserved in his specimens, he considered these clefts to be unique to Giardia among all protozoa and formed by extraction of an unknown component(s) during specimen preparation. Thus the ER in Giardia could have two subregions with different compositions, although both regions contain Bip. The nuclear envelope is considered to be a subregion of ER and we also observed BiP labeling at this site. Cleft regions were also found in the nuclear envelope, suggesting further compositional similarities. These findings of ER subregions and the pervasiveness of ER membranes throughout the cell body are con-

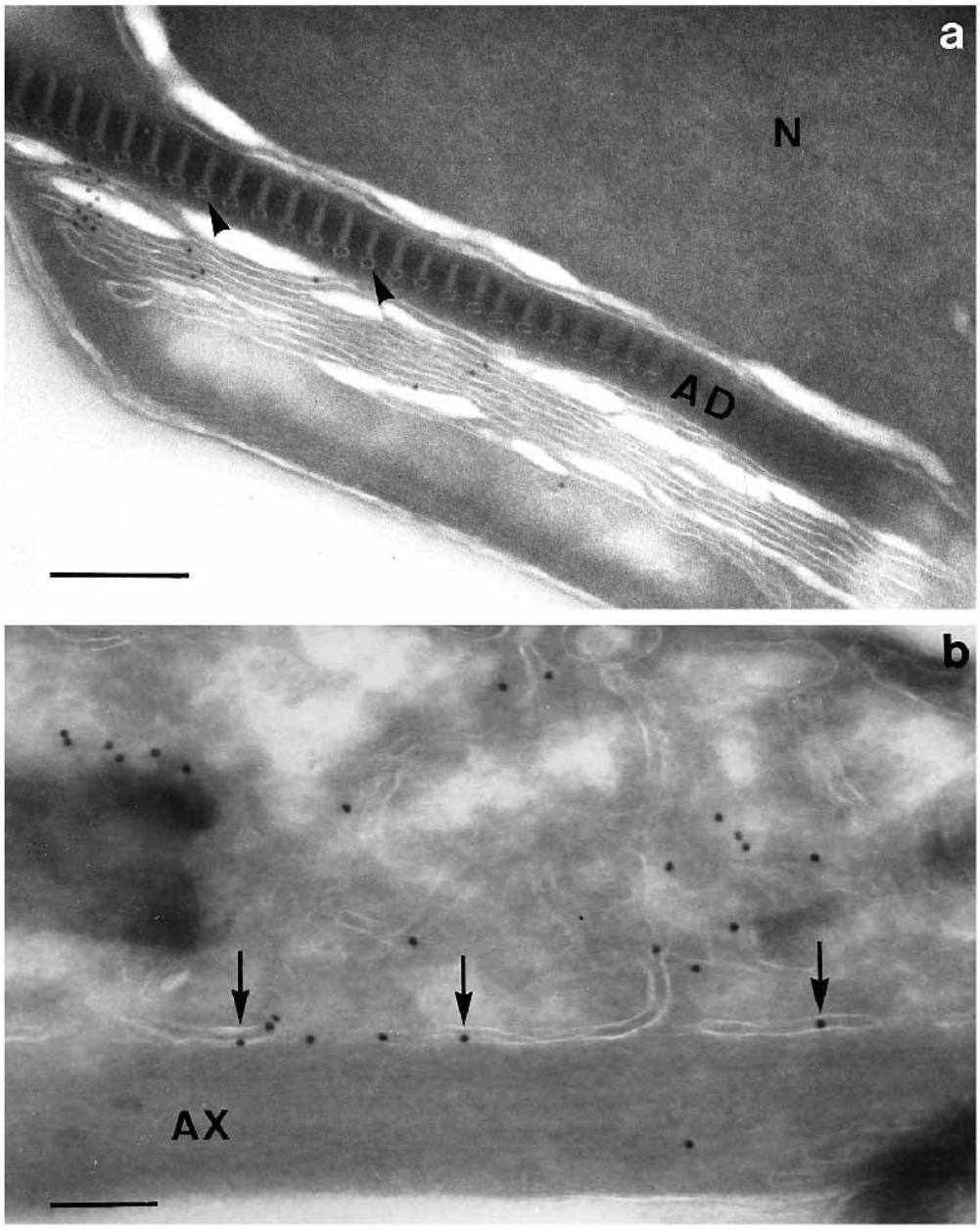

Fig. 7. Bip immunogold labeling of endomembranes associated with microtubule-based structures. (a) Stacked membranes associated with the adhesive disk (AD) in which microtubules (arrowheads) are viewed in cross section. N, nucleus. 10 nm gold labels. (b) ER tubules indicated by arrows are associated with an intracytoplasmic axoneme (AX). 18 nm gold labels. Bars, 0.2 μm.

sistent with current appreciation of the complex organization of ER in higher eukaryotes (Sitia and Meldolesi, 1992).

Our study has been of vegetatively growing trophozoites and in these we have also identified new juxtanuclear membrane stacks which were only partially labeled with BiP antibody. These membranes may represent either annulate lamellae (Kessel, 1992), karmellae (multilamellar ER) (Wright et al., 1988; Wanker et al., 1995) or a post-ER compartment such as the Golgi apparatus. Annulate lamellae are least favoured because the membrane stacks do not have the pore complexes that characterize such structures. If these membranes were a form of karmellae, which appear in cells overproducing ER membrane proteins (Wright et al., 1988; Wanker et al., 1995), then one would expect more uniform Bip labeling, as seen in other ER membranes in Giardia and in previous studies (e.g. Preuss et al., 1991). Alternatively, these flattened cisternae may represent a primitive Golgi apparatus - BiP should localize to both ER and Golgi membranes because Giardial BiP has been shown to contain a KDEL retrieval signal (Gupta et al., 1994) which in higher eukaryotes is recognized by a *cis*-Golgi receptor that functions to send BiP back to the ER (e.g. Lewis and Pelham, 1992). We also found BiP labeling was restricted to particular layers in the juxtanuclear stacks, in contrast with the more consistent labeling of all unstacked ER membranes. Based on the criteria of morphology, subcellular distribution and Bip localization only within a subregion, this membrane system is suggested to be the Golgi apparatus. This putative Golgi apparatus extends over a large area, which is somewhat different from the small compact juxtanuclear mass usually seen in mammalian cells. However, large extensive Golgi systems have also been observed in certain mammalian cells (see Rambourg and Clermont, 1990). Further characterization of these stacked membranes in Giardia trophozoites will require additional molecular markers which are specific for the Golgi (Banting et al., 1995).

ER and Golgi-like membranes may now join peripheral vacuoles and the nuclear envelope (Friend, 1966) as identified endomembranes in Giardia trophozoites. The encystation stage of Giardia's life cycle can be artificially induced in culture (Reiner et al., 1990) and studies of this stage have provided evidence for developmental changes in endomembranes. A Golgi apparatus structure was previously reported to form de novo uniquely during encystation, correlating with biochemical evidence for the appearance of regulated secretion (Reiner et al., 1990; Lujan et al., 1995). The de novo formation of a Golgi apparatus from ER, although possible, is considered a slow process and 'a strong selective disadvantage in a competitive environment' (Warren and Wickner, 1996). Encystation to form a dormant cyst is, in fact, a response to a hostile environment. We speculate that the Golgi apparatus is present throughout Giardia's life cycle but in functionally different forms. McCaffery and Gillin (1994) have also indicated Golgi-like membranes are present in both trophozoites and encysting cells. Encystation-specific vesicles (ESVs) have also been identified in encysting cells - ESVs measure more than 1 μm in diameter and are involved in cyst wall formation (Reiner et al., 1990; McCaffery and Gillin, 1994; McCaffery et al., 1994). How ESVs form is not known. McCaffery and Gillin (1994) have also seen structures resembling transitional elements, putative tubular-vesicular elements and putative transport vesicles. Further molecular marker characterization of Giardia endomembranes, which clearly have a complex organization, should prove interesting from both cell biological and evolutionary perspectives.

In conclusion, the evolution of ER is thought to have taken place in concert with the nucleus (Alberts et al., 1994; Gupta and Golding, 1996) and our findings of ER in the most primitive of known eukaryotes supports this central tenet in cell biology. The structural complexity of the endomembrane system, however, was not anticipated. This is at variance with the suggestion that the earliest eukaryotes possessed an elementary or diminutive endomembrane prototype that later evolved into endomembrane systems with greatly increased complexity.

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