Strategies To Discover the Structural Components of Cyst and Oocyst Walls

John Samuelson,a,b G. Guy Bushkin,a,b* Aparajita Chatterjee,a Phillips W. Robbinusa

Department of Molecular and Cell Biology, Boston University Goldman School of Dental Medicine, Boston, Massachusetts, USA; Department of Microbiology, Boston University School of Medicine, Boston, Massachusetts, USA

Cysts of *Giardia lamblia* and *Entamoeba histolytica* and oocysts of *Toxoplasma gondii* and *Cryptosporidium parvum* are the infectious and sometimes diagnostic forms of these parasites. To discover the structural components of cyst and oocyst walls, we have developed strategies based upon a few simple assumptions. Briefly, the most abundant wall proteins are identified by monoclonal antibodies or mass spectrometry. Structural components include a sugar polysaccharide (chitin for *Entamoeba*, β-1,3-linked glucose for *Toxoplasma*, and β-1,3-linked GalNAc for *Giardia*) and/or acid-fast lipids (*Toxoplasma* and *Cryptosporidium*). Because *Entamoeba* cysts and *Toxoplasma* oocysts are difficult to obtain, studies of walls of nonhuman pathogens (*E. invadens* and *Eimeria*, respectively) accelerate discovery. Biochemical methods to dissect fungal walls work well for cyst and oocyst walls, although the results are often unexpected. For example, echinocandins, which inhibit glucan synthases and kill fungi, arrest the development of oocyst walls and block their release into the intestinal lumen. *Candida* walls are coated with mannans, while *Entamoeba* cysts are coated in a dextran-like glucosyl polymer. Models for cyst and oocyst walls derive from their structural components and organization within the wall. Cyst walls are composed of chitin fibrils and lectins that bind chitin (*Entamoeba*) or fibrils of the β-1,3-GalNAc polymer and lectins that bind the polymer (*Giardia*). Oocyst walls of *Toxoplasma* have two distinct layers that resemble those of fungi (β-1,3-glucan in the inner layer) or mycobacteria (acid-fast lipids in the outer layer). Oocyst walls of *Cryptosporidium* have a rigid bilayer of acid-fast lipids and inner layer of oocyst wall proteins.

Acid-fast lipids,
Fibrils of beta-glucan,
Dityrosine glows.
—Anonymous, Haiku of the oocyst wall

In this minireview, we discuss strategies that have worked well to discover structural components of protist walls that allow them to travel by the fecal-oral route from one person to the next (1–5). Proteins and sugars are the major components of cyst walls of *Entamoeba* and *Giardia*, which cause amebic dysentery and diarrhea, respectively (6–8). In addition to proteins and carbohydrates, lipids are important structural components of oocyst walls of *Cryptosporidium* and *Toxoplasma*, which cause diarrhea and disseminated infections, respectively (9–11). To characterize the structural components of walls of so many different organisms, we have had to focus on what is likely to matter most. Many of these wall-oriented strategies are based upon a set of assumptions that may not be obvious to investigators working on stages of the parasites that are motile and dividing (12). Therefore, we review here the strategies and assumptions most useful to determine the protein, carbohydrate, and lipid compositions of cyst and oocyst walls. The compositions of these cyst and oocyst walls are then used to make simple, if incomplete, models for their structure and assembly.

FIRST STRATEGY: ASSUME THAT AT LEAST SOME OF THE IMPORTANT COMPONENTS OF CYST AND OOCYST WALLS HAVE ALREADY BEEN IDENTIFIED

The life cycle of each parasite reveals important properties of cyst and oocyst walls (Fig. 1) (13–15). Each of *Giardia*, *Entamoeba*, and *Cryptosporidium* has a single walled form, which is infectious and diagnostic. In contrast, *Toxoplasma* has three walled forms (underlined): oocysts shed in cat feces, within which sporocysts form when oocysts sporulate in the environment and tissue cysts in brains and muscles of warm-blooded animals. *Eimeria*, which is a pathogen of chickens, makes oocysts and sporocysts that closely resemble those of *Toxoplasma* (9, 11, 16).

*Giardia* cysts and *Cryptosporidium* oocysts survive for very long periods of time in fresh water, while oocyst walls of *Toxoplasma* resist treatment with 2% sulfuric acid (17). Chitin, which was first identified biochemically in cyst walls of *Entamoeba*, is shown with antibodies to Jessie 3, a chitin-binding lectin (sugar-binding protein) (Fig. 2A) (6, 18). Biochemical studies showed that *Giardia* cyst walls contain fibrils of β-1,3-linked GalNAc, which are detected with antibodies to *Giardia* CWP1, a lectin that binds the GalNAc homopolymer (Fig. 2B) (7, 19). Acid-fast lipids in oocyst walls of *Cryptosporidium* stain with carbol fuchsin, which also stains acid-fast lipids in the walls of mycobacteria (Fig. 2C) (20). The autofluorescence of *Toxoplasma* oocyst and sporocyst walls in UV light indicates the presence of dityrosines, which are formed when Tyr-rich proteins are oxidized (Fig. 2D) (9, 16, 21). Dityrosines are also present in the *Saccharomyces* spore wall (22).

Whether intact, broken by excystation, or disrupted by sonication and/or glass beads, walls of all parasites appear rigid by fluorescence microscopy and transmission electron microscopy (TEM). Sonicated *Giardia* walls shatter and form fragments with...
sharp edges not unlike shards of broken pots (Fig. 2B) (7). The rigidity of the Cryptosporidium oocyst wall is shown by the uniform, oval shape of intact oocysts, the cup-like appearance of excysted walls, and the tight scrolls formed by mechanically broken walls (Fig. 2K) (10). Atomic force microscopy shows that the oocyst wall resembles common plastic materials (23).

TEM reveals the relative simplicity of cyst walls of Giardia and Entamoeba, each of which has a single layer of uniform thickness and staining (Fig. 2E) (24). Similarly, the walls of Saccharomyces and Cryptosporidium, as well as walls of nematode eggs, have multiple layers (Fig. 2G) (10, 16). Since different structural components will likely bind different proteins, one can expect that the protein composition of oocyst walls is much more complicated than that of cyst walls.

A monoclonal antibody identified the major protein of cyst walls of Giardia (CWP1), which has an N-terminal signal peptide, a Leu-rich repeat domain, and a C-terminal Cys-rich domain (Fig. 2B) (25, 26). A monoclonal antibody identified the most abundant protein in Cryptosporidium oocyst walls, COWP1, which contains an N-terminal signal peptide and numerous Cys-rich repeat domains and His-rich domains (27). Monoclonal antibodies also identified Ser- and Thr-rich glycoproteins which are teth-

FIG 1 Cyst and oocyst walls of human pathogens contain a structural sugar polymer (chitin, β-1,3-GalNAc, β-1,3-glucan, or cellulose) and/or acid-fast lipids. Saccharomyces walls contain chitin and β-1,3-glucan. Toxoplasma has three walled forms (oocyst, sporocyst, and tissue cyst). The structural components of tissue cysts of Toxoplasma has not been identified. Bradyzoites are not shown within Toxoplasma tissue cysts. No attempt was made to draw structures to scale.

FIG 2 Fluorescence and transmission electron micrographs (TEMs) show important structural features of cyst walls of Entamoeba and Giardia and oocyst walls of Cryptosporidium and Toxoplasma. (A) Antibodies (red) show Jessie lectins bound to chitin fibrils of the Entamoeba cyst wall. (B) Antibodies (green) show CWP1 in a shard of the cyst wall of Giardia broken by sonication. (C) Carbol fuchsin (red) stains acid-fast lipids in Cryptosporidium oocyst walls. (D) Dityrosines (blue) in oocyst wall (white arrow) and sporocyst walls (blue arrows) of Toxoplasma are autofluorescent in the UV channel. (E) Three Entamoeba cyst walls isolated by centrifugation have uniform thickness and appearance. (F) Recombinant CWP1 binds to fibrils of β-1,3-GalNAc in cyst walls of Giardia deproteinated with NaOH. (G) The Cryptosporidium oocyst wall has a fibrillar glycocalyx (G), a bilayer (Bil), an inner layer containing oocyst wall proteins (OWPs), and tethers (T) that appear globular after they have broken. (H) Recombinant dectin-1 (red) binds to the oocyst wall (white arrow) but not to sporocyst walls of Toxoplasma (same oocyst as shown in panel D). (I) Recombinant Jessie lectin of Entamoeba self-aggregates and forms a biofilm composed of branched fibrils. (J) Deproteinated cyst walls of Giardia form a hollow sphere of curled fibrils of β-1,3-GalNAc. (K) A sonicated and pronase-treated wall of Cryptosporidium contains a rigid bilayer (Bil) and nothing else. (L) A sonicated Eimeria oocyst wall has an inner layer, which is a porous scaffold of fibrils of β-1,3-glucan. Micrographs of Entamoeba are reprinted from references 6 (A and I) and 24 (E), of Giardia from reference 7 (F), of Cryptosporidium from references 10 (G and K) and 11 (C), and of Toxoplasma from reference 9 (D, H, and L). Panels B and J are original here.
TABLE 1 Structural components of fungal, cyst, and oocyst walls

<table>
<thead>
<tr>
<th>Molecule(s)</th>
<th>Structural component(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugar polymer(s)</td>
<td>Chitin, β-1,3-glucan</td>
</tr>
<tr>
<td>Lipids</td>
<td>None</td>
</tr>
<tr>
<td>Proteins</td>
<td>Glucanase, chitinase, dityrosines (spores), ~100 proteins with modified GPI anchors</td>
</tr>
<tr>
<td>Abundant glycans</td>
<td>Mannans (high-mannose N-glycans), Man-rich O-glycans</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Saccharoclycines</th>
<th>Entamoeba</th>
<th>Giardia</th>
<th>Toxoplasma (oocyst)</th>
<th>Cryptosporidium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitin, β-1,3-glucan</td>
<td>None</td>
<td>β-1,3-GalNAc</td>
<td>β-1,3-Glucan Acid-fast lipids</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>Glucanase, chitinase, Jacob lectin, Jessie lectin</td>
<td>None to CWP3 (GalNAc-binding lectins)</td>
<td>None Acid-fast lipids</td>
<td></td>
</tr>
<tr>
<td>Dextran-like O-P-glycans, N-glycans with galactose</td>
<td>Very short N-glycans</td>
<td>GalNAc- and fucose-rich O-glycans</td>
<td>GalNAc- and fucose-rich O-glycans</td>
<td></td>
</tr>
</tbody>
</table>

**SECOND STRATEGY: THE PREDICTED PROTEINS OF EACH ORGANISM SUGGEST IMPORTANT STRUCTURAL COMPONENTS OF THE CYST AND OOCYST WALLS**

Here we assume that fibrils of sugar polymers are major structural components for eukaryotic walls in the same way that peptidoglycans and capsular sugars are major structural components of bacterial walls. Sugar polymers that make fibrils include β-1,3-linked glucan (chitin), β-1,4-linked glucose (cellulose), and β-1,3-glucose (glucan). Cellulose in walls of plants and algae is the most abundant cyst wall protein (Fig. 1). Fibrils of β-1,3-glucan are a major structural component of fungal walls, and the glucan synthase is the target of antifungal drugs called echinocandins (22, 31).

Chitin, cellulose, and β-1,3-glucan can be recognized by the enzymes that synthesize and hydrolyze them. Assuming that each of these enzymes evolved once, one can use homology searches of proteins predicted by whole-genome sequences to determine whether or not a given organism is capable of making each of these sugar polymers (32). Alternatively, one can use a key word search in EuPathDB, but beware that automated annotations may be inaccurate (33). *Entamoeba* is the only protist that makes chitin (6, 18, 34), and *Toxoplasma* and *Eimeria* make β-1,3-glucan (9), while *Acanthamoeba* and *Dictyostelium* make cellulose (Fig. 1 and Table 1) (35, 36). *Cryptosporidium* makes none of these sugar polymers (so how does it make a wall?). These homology searches can correct mistakes resulting from the use of plant lectins that are not always specific. For example, wheat germ agglutinin appeared to identify chitin in cyst walls of *Giardia* and tissue cyst walls of *Toxoplasma*, even though neither parasite has a chitin synthase and so neither can make chitin (37, 38).

If the life cycle of the parasite contains multiple walled forms (e.g., oocysts, sporocysts, and tissue cysts of *Toxoplasma*), the predicted glucan synthases and glucan hydrodolases do not identify which walled stage(s) contains β-1,3-glucan (15). Fortunately, there are multiple glucan-binding reagents (macrophage dectin-1, anti-glucan antibodies, and glucan-binding domains of fungal glucan hydrodolases) that may be used to show that oocyst walls of *Toxoplasma* contain fibrils of β-1,3-glucan whereas walls of sporocysts and tissue cysts do not (Fig. 2D and H) (9, 39).

The enzymes that make and break the unique β-1,3-GalNAc polymer of *Giardia* cyst walls are not homologous to those that make the other sugar polymers and so cannot be discovered by homology searches (19, 32). Similarly, none of the plant lectins that bind GalNAc bind to the β-1,3-GalNAc fibrils in cyst walls of *Giardia*. Instead, the most abundant cyst wall protein of *Giardia* (CWP1) is a lectin that binds to the β-1,3-GalNAc polymer (Fig. 2B and F) (7).

Phylogenetic profiling may be used to search for proteins and/or glycans that are present in coccidian parasites that make oocyst walls and are transmitted by the fecal-oral route (*Cryptosporidium, Toxoplasma, and Eimeria*) but are absent from related apicomplexan hemoparasites, which are transmitted by arthropods (*Plasmodium, Babesia, and Theileria*) (Fig. 3 and Table 1).
Our assumption is that proteins common to coccidia and hemoparasites are unlikely to be part of oocyst walls, while proteins that are specific to coccidia are more likely to be part of oocyst walls. Further, because the proteins share common ancestry, one may assume, until proven wrong, that shared proteins have the same function in the oocyst walls of all coccidia.

Oocyst wall proteins (OWPs), which are the most abundant proteins in Cryptosporidium walls, are also present in the oocyst walls of Toxoplasma and Eimeria (references 40, 41, and 42 and our unpublished data). Polyketide synthases (PKSs) of Cryptosporidium, Toxoplasma, and Eimeria, which are very large enzymes similar to fatty acid synthases, resemble the PKSs that make acid-fast lipids in mycobacterial walls (43, 44). As the oocyst walls of each parasite are acid-fast (Fig. 2C), it is likely that PKSs are involved in synthesis of lipids in oocyst walls (11, 20).

O-GalNAc transferases (GalNACT), which resemble mammalian enzymes, add GalNAc to Ser and Thr of secreted and membrane proteins of Cryptosporidium, Toxoplasma, and Eimeria (45, 46). These O-glycans, which are not stage specific, can be detected with the plant lectin MPA, which binds avidly to oocyst and sporocyst walls (9, 10). Finally, Asn-linked glycans (N-glycans) of coccidia contain 7 to 10 sugars (Glc 0– 3Man5GlcNAc2) (47). In contrast, the N-glycans of Plasmodium and Babesia contain two sugars (GlcNAC2), while Theileria makes no N-glycans. Whether N-glycans with mannose are important for oocyst wall formation has not been determined. In contrast, N-glycan length appears to be irrelevant for cyst walls, as Entamoeba makes N-glycans with Man6GlcNAC2, while Giardia makes N-glycans with GlcNAC2 (48).

Phylogenetic profiling may also be used to identify proteins or glycans shared by Toxoplasma and Eimeria that make oocysts with two walls versus Cryptosporidium proteins that make oocysts with one wall (Fig. 1 and 3). These proteins include enzymes that make or hydrolyze β-1,3-glucon, as well as tyrosine-rich proteins that are oxidized to form dityrosines that autofluoresce in UV (Fig. 2D). In addition, there is a Cys-rich repeat (CRR) protein in oocyst walls of Toxoplasma and Eimeria that binds to deproteinated oocyst walls (our unpublished data).

THIRD STRATEGY: STUDIES OF CYST AND OOCYST WALLS OF NONHUMAN PATHOGENS MAY ACCELERATE DISCOVERY

Entamoeba histolytica, a human pathogen, does not encyst in axenic culture (growth without bacteria) or in the mouse model of intestinal infection. It is therefore necessary to travel to places where ameba infections are common such as India, Bangladesh, or Mexico to collect fecal samples that contain E. histolytica cysts. Alternatively, one can study cysts of E. invadens, an important pathogen of reptiles in zoos, which readily form cysts in axenic cultures (49). All of the abundant proteins in E. invadens cyst walls are present in cyst walls of E. histolytica, and the enzymes that make, degrade, and deacetylate chitin are the same (6, 24, 34, 50–55).

The most likely reason that relatively little is known about oocysts and sporocysts of Toxoplasma is the difficulty associated with infecting kittens with the parasite (4, 15). The life cycle of Toxoplasma in the research laboratory usually involves orally infecting mice with sporulated oocysts and then orally infecting cats with mouse brains containing tissue cysts. Genetic crosses are made by feeding cats brains from mice infected with different strains of Toxoplasma (56). The rapid attenuation of Toxoplasma in culture means that gene knockout technologies, which are used for in vitro cultures and for mouse infections, are not presently available for cat stages of the parasite life cycle (57). For example, the current KU80 knockout strains, like their parent strains, cannot complete their sexual cycle in the cat.

Kittens infected with Toxoplasma do not appear sick but shed tens of millions of oocysts in the stool. Within 48 h in the environment, these oocysts sporulate and become highly infectious. Eimeria provides a safe and relatively inexpensive model for cat stages of Toxoplasma, as oocysts and sporocysts of the chicken parasite closely resemble those of Toxoplasma in morphology and biochemistry (9, 11, 16). Discoveries made with Eimeria in chickens which may contribute to our understanding of Toxoplasma in cats include (i) identification of Tyr-rich proteins in oocyst walls and characterization of the peroxidases that form dityrosines (21), (ii) identification of prominent secretory vesicles (wall-forming bodies) during oocyst wall formation (58), and (iii) development of attenuated strains of Eimeria to vaccinate chickens (59).

FOURTH STRATEGY: TOOLS DEVELOPED TO STUDY YEAST WALLS DEMONSTRATE UNIQUE PROPERTIES OF CYST AND OOCYST WALLS

Biochemical methods for studying yeast walls work well for studying cyst and oocyst walls (22). Like fungal walls, parasite walls can be broken with glass beads and/or sonication and then purified on density gradients (10, 24, 51). Proteins may then be released with trypsin and analyzed with mass spectrometry, using whole-genome sequences of each parasite to predict tryptic peptides (10, 51). N-glycans released with peptide:N-glycanaseF (PNGaseF) and O-glycans released by β-elimination may be digested with glycohydrodrolases and identified by gel filtration or mass spectrometry (48, 51, 60).

The underlying fibrillar structure of the GalNAc homopolymer of Giardia cyst walls is revealed by treatment with NaOH, which deproteinates fungal walls and exposes fibrils of chitin and β-1,3-glucan (Fig. 2F). NaOH-treated walls may be used to pull down proteins from lysed parasites that bind fibrils of the sugar polymer (7). The lectin activities of recombinant cyst or oocyst wall proteins may also be determined using deproteinized walls of parasites or fungi (Fig. 2F) (6–9).

Zymolyase, an extract of Streptomyces that contains proteases and β-glucanases, releases the same glucose disaccharides from fungal walls and oocyst walls of Eimeria (9). Trifluoroacetic acid, which releases glycans in O-phosphodiester-linked bonds to other sugars, also releases Entamoeba sugars linked by O-phosphodiester bonds to Ser and Thr of glycoproteins (51, 61). Nucleotide sugars (e.g., UDP-GalNAc or GDP-fucose) are transported from the cytosol to the lumen of the ER or Golgi apparatus, where sugars are added to secreted and membrane proteins (62). Nucleotide sugar transport assays, first developed with yeast membranes, may be used to identify sugars (e.g., GalNAc or fucose) that each parasite can add to its N-glycans or O-glycans (10).

The function of parasite enzymes may be determined by complementing yeast knockouts (e.g., the Entamoeba chitin synthase 2 complements a Saccharomyces chs1/chs2 double knockout) (34). Finally, organic solvents, which have little effect on fungal and cyst walls, efficiently extract acid-fast lipids from mycobacterial and oocyst walls (11).

While the biochemical methods for investigating fungal and parasite walls are similar, the results with the parasite walls are
Also present in parasite walls (6, 8, 9, 51). Although the chitin of yeast remains in solution or sediments of ancestry and are at different ends of the lectins (24,53). The spacers (pink) has Ser- and Thr-rich regions, which contain O-phosphodiester-linked glycans (51). Acanthamoeba proteins with three cellulose-binding domains (CeBD) have a structure similar to that of the Entamoeba lectins (36). (C) Giardia cyst wall proteins (CWP1 to CWP3) have a Leu-rich repeat (blue-green) that binds fibrils of the β-1,3-GalNAc polymer (7). The Cys-rich region (CRB in pink) is necessary for binding to the wall (70). (D) Toxoplasma and Schizosaccharomyces glucan hydrolases have catalytic domains that share common ancestry (dark blue), but they have glucan-binding domains (GBD) that do not share common ancestry and are at different ends of the lectins (9). (E) The wall of Saccharomyces is covered with mannans, which are glycoproteins containing N-glycans with long mannos chains (Table 1) (22). Cryptococcus is covered by a carbohydrate capsule that is important for pathogenesis (65). While cyst walls of Entamoeba and oocyst walls of Toxoplasma and Cryptosporidium also contain proteins with N-glycans, the mannose chains of these parasites are not nearly as long as those in fungi (47, 48, 60). The most abundant carbohydrate on Entamoeba cysts is a polymer of α-1,6-linked glucose similar to that of bacterial dextran but made by distinct enzymes (51, 61).

In summary, if you know everything about the walls of fungi, you may know a little about the walls of parasites, mainly, the synthesis and hydrolysis of chitin and of β-1,3-glucan.

FIFTH STRATEGY: MAKE MODELS FOR THE STRUCTURE OF CYST AND OOCYST WALLS

Entamoeba. As a starting point, we describe a four-lectin model of the cyst wall of Entamoeba, which we have studied for the longest time and in the most depth (Fig. 1 and 5A) (6, 8, 24, 34, 50–55). Three abundant cyst wall proteins of Entamoeba, each of which was identified by mass spectrometry with excellent coverage, are chitin-binding lectins that cross-link chitin fibrils (Jacob), hydrolyze chitin (chitinase), or self-aggregate (Jessie lectins) and form the daub that holds walls together and makes them impermeable (Fig. 2A and 4A and B and Table 1). The evidence for these conclusions includes (i) demonstration that each cyst wall protein (expressed as a recombinant protein in bacteria or as an epitope-tagged protein in Entamoeba) binds particulate chitin and (ii) use of antibodies to recombinant proteins to confirm their presence in cyst walls (as well as their absence in trophozoites).

Jacob lectins have two to seven tandemly arranged, Cys-rich, chitin-binding domains, which are separated by low-complexity spacers (Fig. 4B) (50). The spacers contain Ser and Thr residues that are decorated with O-phosphodiester-linked, dextran-like glycans (51). The dextran-like glycans of Entamoeba cysts are the same as those on trophozoites, only the reducing end sugar is rhamnose rather than galactose (61). The Jacob lectin spacers also contain sites for cleavage by parasite Cys proteases, which reduce the number of tandemly arranged chitin-binding domains (51). Very similar lectins with sets of Cys-rich, chitin-binding domains, between which are Ser- and Thr-rich spacers, are present in GalNAc homopolymer (Fig. 2B and F and 4C) (7, 25, 26). Cys- and His-rich OWPs are by far the most abundant proteins in oocyst walls of Cryptosporidium, which contain a small number of other possible oocyst wall proteins (POWPs) (10, 27). Remarkably, these OWPs and POWPs are present in just two of ~20 transcription groups identified in Cryptosporidium synchronously forming oocysts in vitro (64). In contrast, oocyst walls of Toxoplasma and Eimeria oocysts, which contain an outer layer with acid-fast lipids and an inner layer with fibrils of β-1,3-glucan, contain dozens of proteins (Fig. 2H and L) (references 9, 11, and 42 and our unpublished data).

Inhibitors of glucan synthases, called echinocandins, are potent drugs for treating disseminated infections with Candida and Aspergillus (31). Echinocandins kill fungi, while these drugs arrest the development of the oocyst walls of Eimeria and block release of the parasites into the intestinal lumen (9). Fibrils of β-1,3-glucan form a dense mesh on the surface of fungi (22). In contrast, fibrils of the same sugar polymer form a porous scaffold in the inner layer of the oocyst wall of Toxoplasma and Eimeria (Fig. 2L) (9).

Almost never what one would predict from the fungal wall model (Table 1). For example, the majority of fungal wall proteins are cross-linked to fibrils of chitin and β-1,3-glucan via sugars remaining in glycosylphosphatidylinositol (GPI) anchors after the lipid portion has been removed (22). As far as we know, none of the parasites use modifications of GPI anchors to cross-link proteins into cyst or oocyst walls.

With the exception of a chitinase in Entamoeba and a glucan hydrolase in Toxoplasma, none of the proteins of fungal walls is also present in parasite walls (6, 8, 9, 51). Although the chitin-binding and glucan-binding domains of the parasite chitinas and glucanases share common ancestry with those of fungi, the parasite carbohydrate-binding domains do not share common ancestry with those of fungi (Fig. 4) (9, 50, 53). While the number of internal repeats in flocculation proteins of Saccharomyces determines whether brewer’s yeast remains in solution or sediments (causing opaque or clear beer, respectively) (63), there appears to be no particular function for the polymorphic internal repeats in spacer regions between chitin-binding domains of Entamoeba Jacob lectins (although these polymorphisms allow us to distinguish clinical isolates by PCR) (52).

While mass spectrometry identifies ~100 proteins in the walls of Saccharomyces, similar methods identify just three proteins in cyst walls of Entamoeba, each of which contains one or more chitin-binding domains (Table 1 and Fig. 2A and 4A and B) (6, 24, 50–52), Giardia also has three abundant cyst wall proteins (CWP1 to CWP3), each of which has a Leu-rich repeat that binds the

![Diagram](http://ec.asm.org/)
sheaths of nematodes and in peritrophic membranes formed by insects around the blood meal (66). Dictyostelium and Acanthamoeba have spore wall proteins that have similar designs, only cellulose-binding domains replace chitin-binding domains (35, 36).

The N-terminal chitin-binding domains of chitinate and Jessie lectin are the same but are distinct from the chitin-binding domains of Jacob lectins or those of other organisms (Fig. 4A) (50). Jessie lectins are secreted after Jacob lectins have cross-linked chitin fibrils and are lectins that bind to curled fibrils of the β-1,3-GalNAc polymer (7, 19, 25, 29). The Cryptosporidium oocyst wall contains a layer of acid-fast fibrils and a layer of oocyst wall proteins (OWPs) (10, 11). The Toxoplasma oocyst wall contains a layer of acid-fast fibrils and a layer that contains fibrils of β-1,3-glucan, oocyst wall proteins, and Tyr-rich proteins that form dityrosines (9, 11, 16, 21).

Because the Jacob and Jessie lectins and chitinate are secreted proteins with no anchor to the plasma membrane, a fourth lectin (the Gal/GalNAc lectin) is necessary to hold the cyst wall to the parasite (24). This Gal/GalNAc lectin, which is an important virulence factor that binds the parasite to host glycans, is expressed on the plasma membrane of trophozoites and cysts (67). The Gal/GalNAc lectin binds to membrane Gln-glycans of cyst wall proteins, so that Entamoeba forms wall-less cysts in the presence of excess galactose.

Two properties of chitin in the Entamoeba cyst wall are of note. First, chitin is made in secretory vesicles that are distinct from those containing Jacob lectins, chitinate, and Jessie lectins (6). In contrast, chitin is made at the plasma membrane of fungi (22). Second, much of the chitin is deacetylated by an Entamoeba chitin deacetylase to form a positively charged species called chitosan (54). Chitosan, which is resistant to hydrolysis by chitinases, is also present in walls of the pathogenic fungus Mucor (68). Finally, the activities of recombinant Entamoeba chitin synthases, chitinases, and chitin deacetylases have all been confirmed by recombinant expression in yeast or bacteria (34, 53, 54).

**Giardia.** The β-1,3-linked GalNAc homopolymer present in the cyst wall of Giardia has not been identified in any other organism (Fig. 1 and 5B and Table 1) (19). Fibrils of the GalNAc homopolymer, which are made in vesicles that are distinct from those containing cyst wall proteins, are curled and form a hollow sphere in the absence of protein (Fig. 2J) (7). Deproteinated walls of Giardia were used to show that native CWP1 and CWP2, which are encystation specific, are lectins that bind fibrils of the GalNAc homopolymer (Fig. 4C). Recombinant versions of CWP1 showed that the Leu-rich repeats at the N terminus rather than the Cys-rich region at the C terminus contain the lectin domain (Fig. 2F). This result was a surprise, as chitin-binding lectins of Entamoeba and insect peritrophins, as well as glucan-binding domains of Toxoplasma glucan hydrolases, are Cys rich (6, 9, 50, 66). On the other hand, Leu-rich repeats present in plant receptor kinases and Toll-like receptors of metazoans bind a wide range of ligands (69, 70).

CWP2 has a very basic tail, which is cleaved by a Cys protease and is deposited along with CWP3 onto fibrils of the GalNAc homopolymer on the surface of encysting Giardia after CWP1 and the rest of CWP2 have been deposited (71, 72). When the Cys-rich region of CWP3 is removed, the protein is secreted but is not retained in the Giardia cyst wall (73). A novel transglutaminase activity is required for cyst wall formation, while Cys protease activity is required for excystation of Giardia (74, 75). There is a Cys-rich repeat protein unrelated to the CWP proteins present in cyst walls of Giardia, and there is massive overexpression of a family of glycine-rich repeat proteins in encysting parasites (76, 77).

While the GalNAc polymer synthase has been enriched in membrane extracts of encysting Giardia, it has not been identified (78). In contrast, the cystolic pathway for synthesis of UDP-GalNAc, the substrate for the GalNAc polymer synthase, has been extensively characterized and shown to be dramatically increased in encysting parasites (79). While many bacteria make glycohydrolases that degrade chitin, cellulose, and/or β-1,3-glucan, no enzymes that degrade the β-1,3-GalNAc have been identified from bacteria or from the host (which has multiple chitinases). Deproteinated walls of Giardia were used to identify a robust cyst-specific glycohydrolase that degrades fibrils of the GalNAc homopolymer (7). This glycohydrolase is not active against intact walls of Giardia, wherein GalNAc fibrils are covered by protein. In contrast, treatment of Giardia cysts with the pancreatic enzyme chymotrypsin exposes GalNAc fibrils and likely contributes to excystation by the parasites in the duodenum.

Wheat germ agglutinin, which mistakenly identifies chitin in Giardia cyst walls, binds to very short N-glycans containing GlcNAc2 (like those present in Plasmodium) (Table 1) (7, 37, 47, 77). These very short N-glycans of Giardia appear to be important for encystation, as morpholinos (small nondegradable DNA homologs) that block N-glycan synthesis cause Giardia to form dysmorphic cysts, often with more than the usual four nuclei (our unpublished data).

In summary, there is a large amount of biochemical detail with regard to cyst wall formation by Giardia, much of which is not readily incorporated into a structural model of the cyst wall.
Cryptosporidium and Toxoplasma. The Cryptosporidium oocyst wall has a glyocalyx on its surface, a rigid bilayer, and an inner layer rich in Cys- and His-rich OWPs (Fig. 2G and 5C) (10). Attached to the inner layer of the oocyst wall are electron-dense globules, which are remnants of Ser- and Thr-rich glycoproteins that tether sporozoites to the oocyst wall. The lectin MPA, which binds O-linked GalNAc, labels very well the outer and inner surfaces of the Cryptosporidium oocyst wall. In contrast, the galactose-binding lectin UEA1 binds only to the inner surface of oocyst walls. While mass spectrometry suggests that OWPs are by far the most abundant proteins in Cryptosporidium oocyst walls, their (self-aggregating, sugar-binding, or lipid-binding) function is not known (10, 27).

Although Cryptosporidium was shown to be acid-fast 30 years ago (when opportunistic Cryptosporidium infection was used as a diagnostic for AIDS prior to the identification of HIV) (20), the function of acid-fast lipids has begun to be understood only recently (Fig. 2C) (11). Acid-fast lipids in Cryptosporidium oocyst walls are part of a rigid bilayer that remains after pronase treatment of oocyst walls (Fig. 2K and 5C) (10, 11). The rigid bilayer is dissolved by organic solvents, and acid-fastness is lost. Similarly, the rigid bilayer and the outer layer of the oocyst wall of Toxoplasma are removed by organic solvents, while the inner layer containing the scaffold of fibrils of β-1,3-glucan remains, for the most part, intact (Fig. 2L and 5D) (11). In contrast, proteases and glucanases dissolve the inner layer of Toxoplasma oocyst walls and leave the outer layer and rigid bilayer intact (9).

Mass spectrometry of oocyst wall lipids released in organic solvents shows triglycerides with elongated fatty acyl chains (in Cryptosporidium) or polyhydroxy fatty acyl chains like those of triglycerides with polyhydroxy fatty acyl chains (in Toxoplasma) (11). Sodium hydroxide cleaves ester linkages in triglycerides and gives a soap bubble appearance to the outer layer of the oocyst wall of Eimeria (a model for Toxoplasma). Finally, a polyketide synthase similar to the enzyme that makes mycobacterial wall lipids is massively overexpressed in oocysts of Toxoplasma and is present in Cryptosporidium (11, 43, 44). Sporocyst walls of Toxoplasma are also acid-fast, suggesting that triglycerides with polyhydroxy fatty acyl chains are important structural components of these walls, which superficially resemble Cryptosporidium oocyst walls (Fig. 1).

Toxoplasma oocyst walls have two features (dityrosines and β-1,3-glucan) found in fungal walls but not found in Cryptosporidium walls (Fig. 5D and Table 1) (9, 10, 16, 21). Dityrosines, which are formed in Toxoplasma when Tyr-rich proteins are oxidized, are present in oocyst and sporocyst walls but not in tissue cyst walls of Toxoplasma (Fig. 2D). The porous scaffold of fibrils of β-1,3-glucan, which is detected with dectin-1, is present in the inner layer of the oocyst walls but is absent from sporocyst and tissue cyst walls (Fig. 2H). A Toxoplasma glucan hydrolase, which has a unique Cys-rich, glucan-binding domain at its N terminus, is present in the inner layer of the oocyst wall (Fig. 4D) (9). A Schizosaccharomyces glucan hydrolase has at its C terminus a glucan-binding domain which does not share common ancestry with that of Toxoplasma.

Treatment of Eimeria-infected chickens with echinocandins that inhibit fungal glucan synthases arrests oocysts in a wall-less form that is not released into the lumen of the ceca (9, 31). Whether echinocandins also affect acid-fast lipids has not yet been determined. These results show that the Eimeria glucan synthase is druggable and that glucans are essential for development of the oocyst wall. The absence of β-1,3-glucan from tissue cyst walls suggests that echinocandins cannot be used to treat residual tissue cysts from humans infected with Toxoplasma.

Toxoplasma oocyst walls contain many more proteins than oocyst walls of Cryptosporidium (Table 1) (references 10 and 42 and our unpublished data). Toxoplasma oocyst wall proteins include Cys- and His-rich OWPs similar to those of Cryptosporidium oocyst walls and Tyr-rich proteins similar to those of Eimeria oocyst walls (21, 40–42). A Cys-rich repeat protein which is also present in Eimeria oocyst walls binds to deproteinized oocyst walls, suggesting that it is either a lectin or a lipid-binding protein (our unpublished data). Ser- and Thr-rich proteins in oocyst walls of Toxoplasma resemble Ser- and Thr-rich proteins present in tethers that attach sporozoites of Cryptosporidium to the inner layer of the oocyst walls (reference 10 and our unpublished data). Like oocyst walls of Cryptosporidium, Toxoplasma oocyst walls label with lectins that bind GalNAc (MPA) and fucose (UEA1) (Table 1) (9–11).

Although β-1,3-glucan is present in oocyst walls but absent in sporocyst walls of Toxoplasma, there is likely considerable overlap of proteins in the oocyst and sporocyst walls that look similar to each other and are both acid-fast (Fig. 2H) (9, 11). In contrast, there is likely little commonality between oocyst walls of Toxoplasma and tissue cyst walls, which do not look similar by TEM, do not have similar staining properties, and do not share many proteins (according to gene expression studies at ToxoDB) (15, 16, 33, 40–42). While fluorescence microscopy, expression studies, and knockouts have suggested a number of important proteins of Toxoplasma tissue cysts (sugars bound by dolichos lectin, bradyzoite-specific antigens, and rhoptry proteins), there is presently no detailed model of the structure of tissue cyst walls, and no sugar polymers or acid-fast lipids have been identified (Fig. 1) (12, 38, 56, 57, 80–84).

In summary, no sugar polymer has been identified in the Cryptosporidium oocyst wall and the Toxoplasma sporocyst wall, each of which contains acid-fast lipids that appear to be a major structural component. Oocyst walls of Toxoplasma contain Tyr-rich proteins with dityrosine cross-links and fibrils of β-1,3-glucan, which are important structural components of fungal walls, as well as acid-fast lipids that are major structural components of mycobacterial walls.

CONCLUSIONS AND FUTURE STRATEGIES

Important strategies to determine the structure of cyst and oocyst walls derive from a few core ideas. TEM shows whether a parasite wall is relatively simple (Entamoeba and Giardia) or complex (Toxoplasma and Cryptosporidium). Each parasite wall has a major sugar polymer (chitin, cellulose, β-1,3-glucan, or β-1,3-GalNAc) or, in rare instances, acid-fast lipids. Monoclonal antibodies and mass spectrometry identify the most abundant proteins, many of which are lectins that bind the sugar polymers. Advantage may be taken of nonhuman pathogens (Entamoeba invadens and Eimeria) to better understand walls of Entamoeba histolytica and Toxoplasma, respectively. The biochemical methods used to study parasite walls are the same as those used to study fungal walls, although the results are usually different. We look forward to the time when a sixth strategy (targeted knockouts and forward genetics) can be applied to studies of cyst and oocyst walls. This is already the case for tissue cysts of Toxoplasma and may soon be the case for Entamoeba cysts and Eimeria oocysts (56, 57, 80, 84–86).
Then we will be able to test the structural models of the cyst and oocyst walls derived from identification of their major components reviewed here.

Finally, there are reasons to be cautiously optimistic that studies of the cyst and oocyst walls may lead to new diagnostics and/or therapeutics for some of these parasites. Already, diagnostic antibody targets protein in the cyst wall of Giardia and the oocyst wall of Cryptosporidium (25, 27, 29, 30). Jacob and Jessie lectins, which are abundant proteins in the amebic cyst wall, are excellent targets for new diagnostic antibodies for Entamoeba (6, 24, 51, 52, 55). Oral glucan synthase inhibitors might be developed to reduce transmission of Eimeria in chickens and Cryptosporidium in cattle using drugs that target synthesis of acid-fast lipids (11). Advantage might be taken of the new culture techniques for Eimeria and Cryptosporidium, which make it possible to screen drugs for their effects on oocyst wall development in vitro rather than in whole animals (87).

ACKNOWLEDGMENTS

We appreciate the many contributions of graduate students and post-docs in the Samuelson and Robbins laboratories, as well as those of collaborators.

This work was supported in part by NIH grants A148082 and A144070 (to J.S.), A107642 (T32 to G.G.B.), and GM31318 (to P.W.R.) and by a grant from the Mizutani Foundation for Glycoscience.

REFERENCES


