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Invited review

The cytoskeleton of *Giardia lamblia*

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Abstract

Giardia lamblia is a ubiquitous intestinal pathogen of mammals. Evolutionary studies have also defined it as a member of one of the earliest diverging eukaryotic lineages that we are able to cultivate and study in the laboratory. Despite early recognition of its striking structure resembling a half pear endowed with eight flagella and a unique ventral disk, a molecular understanding of the cytoskeleton of *Giardia* has been slow to emerge. Perhaps most importantly, although the association of *Giardia* with diarrhoeal disease has been known for several hundred years, little is known of the mechanism by which *Giardia* exacts such a toll on its host. What is clear, however, is that the flagella and disk are essential for parasite motility and attachment to host intestinal epithelial cells. Because peristaltic flow expels intestinal contents, attachment is necessary for parasites to remain in the small intestine and cause diarrhoea, underscoring the essential role of the cytoskeleton in virulence. This review presents current day knowledge of the cytoskeleton, focusing on its role in motility and attachment. As the advent of new molecular technologies in *Giardia* sets the stage for a renewed focus on the cytoskeleton and its role in *Giardia* virulence, we discuss future research directions in cytoskeletal function and regulation.
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1. Introduction

The beauty of the *Giardia* cell has captured the imagination of biologists since the late 1600's when Anton van Leeuwenhoek is believed to have first seen it (Dobell, 1920). Leeuwenhoek's anecdotal linkage of the *Giardia* in his stool with an ongoing bout of diarrhoea was subsequently confirmed in principle by Lambl in the mid-1800's (Lambl, 1859). Indeed *Giardia lamblia* (alternatively referred to in the literature as *Giardia intestinalis* and *Giardia duodenalis*) is one of the most ubiquitous intestinal pathogens, capable of infecting a wide range of mammals including humans, agriculturally important livestock such as cattle and sheep, and a broad array of reservoir hosts in the wild. Additionally, other *Giardia* species infect fish, amphibians, reptiles, birds, reptiles, and rodents.

In *Giardia* there is apparently a strong link between the cytoskeleton and virulence. Virulence requires a pathogen to enter a host, maintain an infection (find the resources to replicate while avoiding destruction by host immune factors), and secure a means of transmission to the next host. For *Giardia*, maintaining an infection relies upon the ability of the parasite to survive in the small intestine of the host. Because this locale is rich in nutrients and relatively tolerant of the presence of microbes, perhaps the greatest challenge facing *Giardia* is peristalsis, the contraction of smooth muscles that propels intestinal contents forward. *Giardia* must be able to manoeuvre within the intestine to find an optimal microenvironment and attach to the epithelial cells to avoid being swept away. It is unclear how, or even whether, *Giardia's* attachment to intestinal epithelial cells results in diarrhoea. Various models have been proposed, including reduction of absorptive surface area or altered cationic/fluid/metabolite balance by crowding, damage to enterocytes by attachment processes or toxins, and immune-mediated pathology (reviewed in Farthing, 1997). Parasites do not invade the host epithelium, but attachment produces ultrastructural changes in the intestinal epithelial cells, most notably as an altering of the shape of the microvilli and destruction of the microvilli at the sites of attachment, presumed to be important in the diarrhoea that sometimes accompanies infection. Although in numerous other intestinal pathogens attachment and consequent host cell damage is mediated by protein-protein interactions with the pathogen's protein termed a virulence factor, in *Giardia*

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attachment appears to be predominantly mediated by the cytoskeleton. It is therefore appropriate to view the cytoskeleton as a potential virulence determinant in *Giardia*. The multiple roles of the cytoskeleton of *Giardia* described in this review – attachment, motility and replication underscores the need for a better understanding of its structure, function and regulation.

2. The basic biology of *Giardia*

An excellent up-to-date review of the biology of *Giardia* is available (Adam, 2001), so only the most salient points are discussed here. *Giardia* belongs to the family Hexamitidae within the order Diplomonadida. The unifying feature within this order, as implied by the name, is the presence of two nuclei. Phylogenetic analyses of sequences of 16S rRNA and numerous protein-coding genes place *Giardia* and the Hexamitidae along the earliest well-characterised branch to diverge from the eukaryotic lineage (reviewed in Adam, 2000). Assuming that the cytoskeleton of *Giardia* is like that of more recently diverged eukaryotes is tenuous due to the large evolutionary separation between *Giardia* and these other organisms.

Giardia has a simple life cycle that alternates between an infectious cyst stage and the intestinal trophozoite stage (reviewed in Gillin et al., 1996; Lujan et al., 1997; Adam, 2001). *Giardia* is most commonly ingested in the form of cysts from contaminated water supplies, though food and direct contact with faecal matter also provide avenues for infection. In vitro studies relying on acidic pH and proteases to initiate excystation suggest that *Giardia* passage through the stomach of its host effects transformation into the trophozoite. The trophozoite then replicates within the lumen of the small intestine, resisting expulsion by adherence to the intestinal epithelium. Infections range from asymptomatic to severe diarrhoea accompanied by nausea, vomiting and gas. The associated malabsorption of nutrients is seen to adversely affect the host as demonstrated through numerous human and livestock studies. The activators for encystation are still controversial. A role for bile is indicated although it remains unclear whether bile acts directly as a signal or indirectly through lipid sequestration.

Reproduction in *Giardia* appears to be limited to fission, as the identification of clonal populations in the wild suggests that sexual reproduction in *Giardia* is either exceedingly rare or absent (Tibayrenc et al., 1990). *Giardia* trophozoites are binucleated and previous work has shown that nuclear division during fission is slightly asynchronous (Wiesehahn et al., 1984). Two recent elegant studies have shed light on the cell cycle, showing: (1) confluent *Giardia* cultures in vitro contain a replicated DNA content, indicating that unlike most other cell types, *Giardia* rests in the G2 phase of the cell cycle (Bernander et al., 2001); and (2) that each daughter cell receives a copy of each of the two nuclei from the parent cell (Yu et al., 2002). The best evidence points towards a tetraploid genome, with debate concerning the distribution of the chromosomes between the two nuclei (Yang and Adam, 1994; Le Blancq and Adam, 1998; Frisardi, M., Samuelson, J., 1999. Each nucleus of *Giardia lamblia* is a clonal lineage. Tenth Annual Molecular Parasitology Meeting, Abstract 263A, Woods Hole, MA; Yu et al., 2002). The *Giardia* genome, like that of many other parasitic protists, is quite ‘plastic’ with frequent expansions, contraction and rearrangements of chromosome ends (Le Blancq and Adam, 1998).

Giardia trophozoites have several unusual organelle features, including an absence of mitochondria and peroxisomes (perhaps not surprising given the anaerobic environment within the small intestine), and a developmentally regulated rough endoplasmic reticulum and Golgi that proliferate during the massive secretion required to build a cyst wall during encystment (Lujan et al., 1995; and reviewed in Gillin et al., 1996; Lujan et al., 1997). Aside from the two nuclei, however, perhaps the most striking feature of *Giardia* is the presence of a complex and unique cytoskeleton. *Giardia lamblia* is a highly polarised protist, and the cytoskeleton is dominated by several structures, including eight flagella, the ventral disk, and median body.

3. The eukaryotic cytoskeleton

The cytoskeleton is now considered to be the key defining feature of eukaryotic cells, preceding and enabling the acquisition of the nucleus in early eukaryotic evolution. The cytoskeleton can best be divided into four basic components: (1) microtubules comprised of tubulin and associated proteins; (2) microfilaments comprised of actin and associated proteins; (3) intermediate filaments and associated proteins; and (4) motor proteins associated with microfilaments or microtubules. Far from existing as separate entities as once assumed, more recent data have pointed to the many connections and interactions among the three classes of fibres.

Microtubules are 25 nm cylinders formed from heterodimers of α- and β-tubulin (reviewed in Cleveland, 1999). In most cells α- and β-tubulin exist as multigene families with different isotypes often serving distinct functions. Microtubule associated proteins also play roles in the assembly/disassembly and function of microtubules. Among the microtubule associated proteins are several newly identified forms of tubulin (γ, δ, ϵ). γ-tubulin is notable as a major component of microtubule organising centres. Microtubules are important in cell motility through their presence in cilia and flagella, in cell division as part of spindle fibres, and in establishing cellular architecture. The two classes of motor proteins associated with microtubules are kinesins and dyneins.

Free actin monomers (G-actin) in the cell assemble head to tail into microfilaments (F-actin); actin is the major protein in the 5–9 nm diameter microfilaments. There are

numerous protein classes of actin-associated proteins, with seven major groupings: monomer binding proteins, small-severing proteins, side-binding proteins, cross-linking proteins, membrane associated actin-binding proteins, microtubule binding proteins, and motor proteins (reviewed in Pollard, 1999). Many of these classes of actin-associated protein are uncharacterised in early diverging eukaryotes. The motor proteins associated with actin are the myosins, a large family of proteins with up to 16 different classes in certain cell types.

Intermediate filaments are 10 nm in diameter and are found in metazoan cells where they contribute to cell structure and strength (reviewed in Goldman and Chao, 1999). Examples of intermediate filament proteins include lamins supporting the nuclear membrane. No intermediate filaments have been described in protists.

4. The *Giardia* cytoskeleton

The introduction in the last 5 years of molecular tools in *Giardia* combined with the new capabilities of microscopy places us in a compelling position to understand the unique nature of the cytoskeleton in a cell that is not only one of the world's leading pathogens, but also the earliest diverging eukaryote capable of being routinely cultured in the laboratory. The study of the cytoskeleton deserves careful molecular analysis considering the enormous morbidity toll that *Giardia* exacts on people and livestock as one of the most common intestinal protist infections in the world. This threat has also recently been recognised in the United States by the naming of *Giardia* as a Category B bioterrorism agent. In addition to the role of the cytoskeleton in virulence described above, novel cytoskeletal proteins provide researchers with potential drug targets for treatment of giardiasis. Unmistakably, the cytoskeleton is responsible for unique *Giardia* structures and adaptations to a parasitic lifestyle. Yet to date our understanding of the *Giardia* cytoskeleton is largely inferred from ultrastructural studies. The key structures of the *Giardia* cytoskeleton were carefully elucidated in a series of biochemical and microscopic studies from the 1950's through the 1980's. Classic biochemical studies by Holberton and colleagues identified some cytoskeletal components, and this work importantly developed methods for isolating cytoskeleton sub-fractions. However, this early work has not been vigorously pursued, and today few molecular players are known or localised. Significantly, there is no empirical evidence of in vivo protein function. With the recent developments of reverse genetics tools and a virtually completed genome, we now have the capability to query protein behaviour and function in live cells. Not only will improved understanding of cytoskeleton-mediated mechanisms facilitate an improved understanding of giardiasis, but it will also afford a unique insight into evolution of the eukaryotic cytoskeleton due to *Giardia's* basal phylogenetic position.

A comprehensive review of the *Giardia* cytoskeleton was last published in 1984 in the book *Giardia and Giardiasis*, edited by S.L. Erlandsen and E.A. Meyer. The chapters 'Structure of the Trophozoite and Cyst' by Dennis E. Feely, Stanley E. Erlandsen, and David G. Chase (Feely et al., 1984) and 'Trophozoite Motility and the Mechanism of Attachment' by Stanley E. Erlandsen and Dennis E. Feely (Erlandsen and Feely, 1984) provide a beautifully illustrated description of the *Giardia* cytoskeleton and propose insights into its function during motility and attachment as was understood at that time. Much of their data, although it is almost 20 years old, still represent the limits of our knowledge in this field, and many of their ideas are consistent with more recent data. We will therefore not use this review to recapitulate their work and simply direct the interested reader to the original. We describe only briefly the basic structural features of the *Giardia* cytoskeleton that have been understood for these past 20 years to lay the groundwork for newer evidence and insights gained from more recent biochemical and molecular studies.

In this review we examine our knowledge about the *Giardia* cytoskeleton considering four levels: structure, protein composition, function, and regulation. Because of the complexity of cytoskeleton studies, we start by describing methodology and tools available for research in *Giardia*. Because a thorough understanding of structure facilitates experiments into function, we then move on to discuss and illustrate the basic structures that comprise the *Giardia* cytoskeleton, with the caveat that most of the attention has focused on the novel structures of the ventral disk and median body, with relatively scant attention paid to other elements. We then discuss what is known about the proteins of the cytoskeleton – localisation and function. Although an understanding of cytoskeleton structure-function relationships in *Giardia* is still rudimentary, many researchers have investigated motility and attachment processes in *Giardia* in the past 2 decades, and we next discuss the interesting, but often contradictory, findings concerning the role of the cytoskeleton in these processes. We conclude by proposing several key challenges for researchers in this field.

5. Methodology and tools

Giardia is particularly well-suited to studies of the cytoskeleton because of its ease of growth and its attachment in a mostly uniform orientation to substrates which facilitates microscopic analysis. In addition, molecular analysis is facilitated by the development of standardised methods for biochemical analysis, the availability of molecular tools for reverse genetic manipulations of the parasite, and a nearly completed genome project.

5.1. Giardia culture and encystation

The cultivation of *Giardia* trophozoite stage parasites in

vitro is safe and straightforward following the method of Keister (1983). *Giardia* typically grows adherent to a density of $1–2 \times 10^6$/ml anaerobically at 37°C in glass screw-capped culture tubes, while the more substantial amounts of material required for biochemical studies can be obtained using tissue culture flasks chosen to maximise surface area to volume ratios. Protocols for in vitro encystation are routine and can be used to achieve up to 50% encystation over a 24 h period (Gillin et al., 1987, 1989). Excystation can also be stimulated in vitro but at much lower efficiencies (~5–10%) (Hetsko et al., 1998; Bernander et al., 2001). Both encystation and excystation are semi-synchronous events in vitro (Bernander et al., 2001).

5.2. Microscopy

Using their ventral disks and flagella, *Giardia* trophozoites adhere to glass slides primarily with the dorsal side up. Although this behaviour permits orientation of cellular structures, it must be noted that a significant minority of parasites (~25%) appear in other orientations in cells fixed to slides (Yu et al., 2002). It is therefore imperative that proper orientation of the parasites be confirmed prior to interpretation of data. Fixation of parasites to preserve both structure and antigenicity is optimally performed with formaldehyde fixation and saponin permeabilisation as is standard methodology for many cell types. Methanol/acetone fixation – commonly used in the literature for fixation of *Giardia* to visualise surface proteins (e.g. Aggarwal and Nash, 1988) should be used with caution. Our own experiments with several monoclonal antibodies suggests that the ventral disk and median body become 'sticky' using this method of fixation and can lead to inaccurate staining – particularly of the disk and median body (unpublished data); additionally, three-dimensional cell structures tend to be flattened with methanol/acetone rendering differential interference microscopy (DIC) imaging and determination of cell orientation difficult.

Electron microscopy and immunoelectron microscopy have been very successful in *Giardia* with numerous published protocols (see for example Feely et al., 1984; Reiner et al., 1990; McCaffery et al., 1994; McCaffery and Gillin, 1994).

Recent improvements in low light cameras have also made possible high-resolution video imaging of parasite flagellar function (Ghosh et al., 2001; Campanati et al., 2002). Although much can be learnt through simple DIC microscopy, future development of green fluorescent protein (GFP)-tagged proteins will be critical in discerning the movement of fine structures during dynamic cellular processes.

5.3. Antibodies

The evolutionary divergence of *Giardia* has resulted in fairly dissimilar protein sequences compared with homologs from other eukaryotic cells. Thus heterologous antibodies (those raised against a different organism's version of the protein) must be used with caution. Additionally, many laboratory rabbits are chronically infected with *Giardia*, and rabbit sera must therefore be carefully pre-screened to ensure that the animals are not already generating a non-specific anti-*Giardia* response. And finally, the common observation that even monoclonal antibodies are often not mono-specific for a single protein holds true in *Giardia* as well: repetitive protein sequences (e.g. the coiled-coil domain common to many cytoskeleton proteins) can be a common antibody target, and a monoclonal antibody that recognises an epitope in a coiled-coil domain is likely to react non-specifically with numerous proteins.

5.4. Cytoskeleton biochemistry

Holberton's laboratory (Holberton and Ward, 1981; Crossley and Holberton, 1983) published cytoskeleton preparation protocols in the 1980's that have been used with subsequent success by several laboratories (Weber et al., 1996, 1997; Nohynkova et al., 2000). Briefly, cytoskeletons are prepared by resuspending cells in 150 mM KCl (or alternatively 150 mM PIPES), 10 mM Tris pH 8.3, 2 mM EDTA, 2 mM $MgSO_4$, 2 mM ATP, 0.5–1.0% Triton X-100 for 1–2 min at room temperature with vortexing. Sufficient quantities of cell material have been obtained to perform protein purification and mass spectrometry on α- and β-tubulin following cytoskeleton preparations (Weber et al., 1996, 1997). Flagella can also be isolated from *Giardia* using a protocol from Holberton (Clark and Holberton, 1988). In short, cells are disrupted by a Polytron tissue homogeniser in a 30 mM Tris, 2.5 mM $MgSO_4$, 200 mM sucrose, 25 mM KCl, pH 7.4, for 1–2 min until flagella dissociate as judged by phase contrast microscopy; flagella can then be further purified on a 40% Percoll gradient. *Giardia lamblia* is quite rich in proteases – particularly cysteine, serine, and aspartic proteases, and aminopeptidases (Hare et al., 1989; Williams and Coombs, 1995). All experiments must therefore be conducted in the presence of protease inhibitors.

5.5. Transient and stable transfections

The ability to express tagged and heterologous proteins in *G. lamblia* is an essential tool for the in vivo study of protein function. In the WB strain of *G. lamblia* (used by many groups for cell biology studies and by the *Giardia* genome project), foreign DNA can be introduced transiently by electroporation and subsequently selected for and maintained using selection with puromycin or G418 either as multicopy episomes or as single copies integrated via homologous recombination (Singer et al., 1998; Sun et al., 1998; and reviewed in Davis-Hayman and Nash, 2002). Of particular importance for studies of cytoskeleton proteins, sequences can be tagged with GFP or short epitopes and localised in living or fixed cells, respectively (Elmendorf

et al., 2000; Hehl et al., 2000). In the future, the use of GFP-tagged cytoskeleton proteins will be essential to studies of cytoskeleton dynamics in *Giardia*.

Ideally investigation of protein function in vivo would examine the phenotype of cells depleted of a single protein to permit linkage of protein to function. However, because of the polyploid genome and lack of sexual reproduction, gene 'knock-outs' are not plausible. Thus, the development of antisense RNA technology to inhibit specific gene expression in *Giardia* is of central importance (Dan et al., 2000; Dan and Wang, 2000). Using antisense constructs (typically longer than 1 kb) incorporating a hammerhead ribozyme, gene expression of the targeted enzymes were inhibited >80%. This reduction of protein levels was sufficient to confirm in vitro analysis of the enzymes studied and attribute biological functions to the activities. Similar studies on cytoskeleton-related proteins should therefore now be possible.

A number of promoters have been described, including the very strong α2-tubulin promoter and the significantly weaker δ-giardin and actin promoters (Elmendorf et al., 2001) that can be used to direct varying levels of heterologous protein expression. This will be critical to studies involving GFP or epitope-tagged proteins because overexpression of proteins has been frequently observed to result in their mislocalisation. Furthermore, three encystation-specific promoters have been characterised (Knodler et al., 1999; Hehl et al., 2000; Davis-Hayman and Nash, 2002) to permit studies on changes in localisation and function throughout the developmental cycle. Experiments using potentially lethal constructs (e.g. antisense or dominant negative mutants) can be performed using a tetracycline-based inducible system developed in *G. lamblia* (Sun and Tai, 2000).

5.6. Genome project

A genome project is underway for *G. lamblia*, with a goal of providing deeper insights into its pathogenic mechanisms, evolution, and genomic organisation (McArthur et al., 2000) (*Giardia* Genome Project http://www.mbl.edu/*Giardia*). As of the April 2002 release, the shot-gun method of sequencing has generated ~83 Mb of sequence – over 11-fold genome equivalents – with estimates of >98% coverage of the genome. Contig assembly is also now underway. Raw data and contigs are made available to researchers as they are obtained.

5.7. Structures

The basic morphology of the *Giardia* cytoskeleton has been delineated through light and electron microscopy studies (for example see Filice, 1952; Friend, 1966; Holberton, 1973a,b, 1981; Erlandsen and Feely, 1984; Feely et al., 1984). Larger and unique structures have been described in detail (especially the ventral disk by Holberton's laboratory), but smaller structures are underrepresented in the literature and a three-dimensional perspective of the *Giardia* cytoskeleton is not available. And although more recent work has elucidated several protein constituents of the cytoskeleton, the majority of proteins remain anonymous, and the structures are known only by vaguely descriptive names – e.g. the dense fibrous rods.

The morphology of *Giardia* when viewed by scanning electron microscopy appears as a flattened pear half, with the larger end of the pear the anterior end of the cell and the rounded side of the pear the dorsal surface of the parasite. Ventral views have shown that this surface of *Giardia* is covered in the anterior half of the parasite with a structure variously termed the 'ventral disk' or 'adhesive disk' unique to *Giardia*; we use the former term here to eliminate mechanistic implications. The cell body extends over the edges of the disk on the first half of the parasite in a flattened protrusion termed the 'ventrolateral flange'. Just posterior to the ventral disk is the 'median body', an array of microtubules also unique to *Giardia*. Four pairs of flagella originate from basal bodies slightly anterior to the two nuclei (see schematic diagram of the cell in Fig. 1). Although the anterior half of the cell is relatively rigid with the ventrolateral flange often caught in scanning electron microscopy in an upward lift, the dorsal end of the parasite is capable of flexion in both the lateral and dorso-ventral orientations (Owen, 1980; Erlandsen and Feely, 1984; Campanati et al., 2002).

Fig. 2 shows several different sections through the parasite and illustrates various features of the cytoskeleton. Fig. 2A is a transverse section of the trophozoite with the dorsal side of the parasite up and the aligned peripheral vacuoles

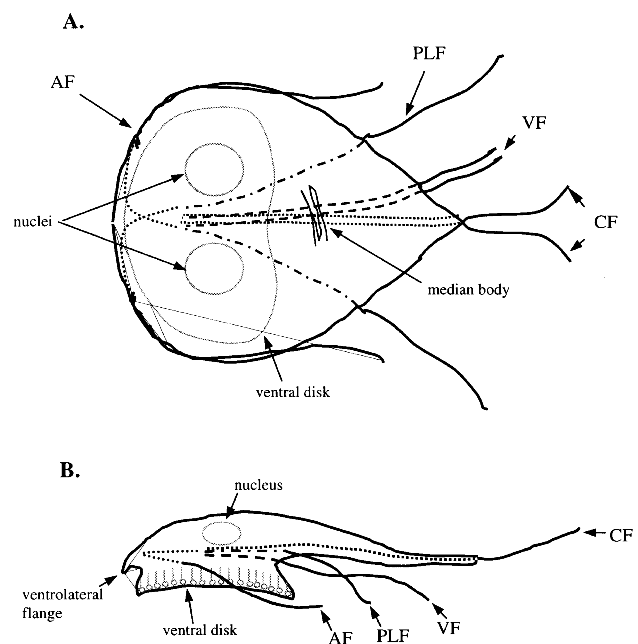

Fig. 1. Schematic representation of key structural features of a *G. lamblia* trophozoite. (A) The parasite is viewed dorsally, with lighter and dotted lines indicating internal structures. (B) The parasite is viewed laterally through the ventral groove. AF, anterior flagella; CF, caudal flagella; PLF, posterior lateral flagella; and VF, ventral flagella.

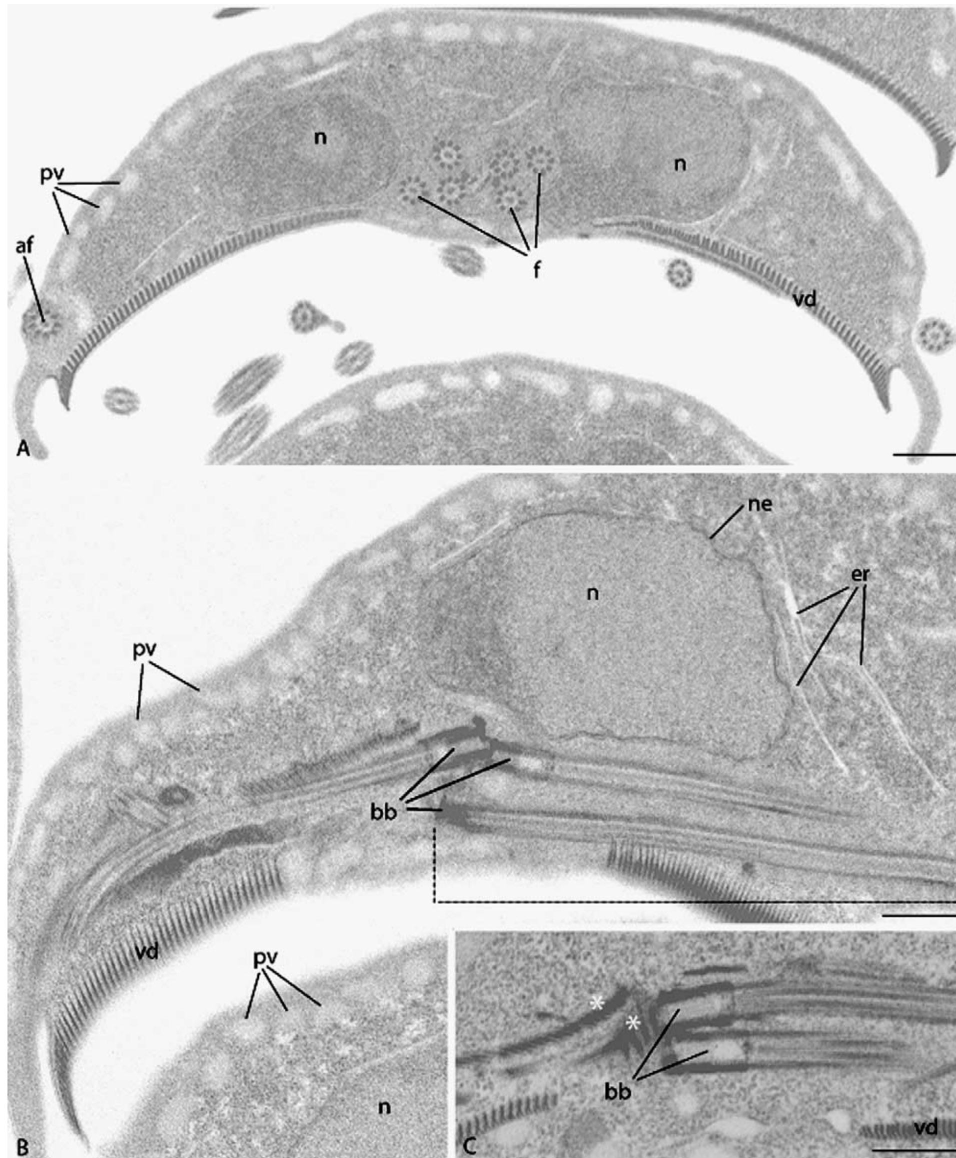

Fig. 2. Conventional TEM sections through *G. lamblia* trophozoites. (A) Typical transverse section profile showing typical intracellular organisation of two nuclei (n); six, central and two anterior flagella (f, af); array of peripheral vacuoles (pv) underlying the plasma membrane; and the ventral disc (vd) denoted by the conspicuous parallel arrays of microtubules. (B) Sagittal sections through the anterior half of the trophozoite revealing typical basal body (bb) and axonemes (dashed line). Also seen is the discontinuous ventral disk (vd); peripheral vacuoles (pv); endoplasmic reticulum (er); and double membraned nuclear envelope (ne). (C) Higher magnification view of basal body/axoneme demonstrating the dense rods (asterisks) arrayed next to the basal bodies (bb). Bars = 0.1 μm.

conspicuously seen just beneath the dorsal surface. The two nuclei are clearly visible containing slightly more electron-dense material than the surrounding cytoplasm; while the ventral disk appears as a comb-like structure on the anterior face of the plasma membrane with protrusions of the cell body (the ventrolateral flange) extending at the sides. The axonemes of the six more posterior flagella are visible between the two nuclei and the two more anterior flagella are visible emerging from the cell body to the left and right of the cell. Figs. 2B,C are sagittal sections through the anterior half of the parasite. In Fig. 2B, the dorsal membrane is again seen with underlying vacuoles while a single nucleus, delimited by the double membrane nuclear envelope, is visible. The ventral disk shows the typical central discontinuity (the 'bare zone'). Axonemes of three flagella are seen in longitudinal section as they originate at the anterior end of the nucleus from typical basal bodies and extend through the cell body. Visible in Fig. 2C are the 'dense fibrous rods' present in close proximity/association with the flagellar basal bodies.

5.8. Flagella

Giardia has eight flagella, arrayed in four pairs termed the

anterior, posterior-lateral, caudal and ventral. The axonemes of these flagella appear to be typically eukaryotic with a $9 + 2$ arrangement of microtubules, and they originate from basal bodies located between and slightly anterior to the two nuclei (see Figs. 2A,B). The anterior flagella extend toward the anterior end of the cell, cross, and then loop within the ventral flange to emerge from the cell body streaming in the posterior direction. The caudal flagella run the length of the cell body, extending from the posterior tip of the cell in a surprising variety of different lengths. The ventral flagella emerge from the ventral side of the parasite just posterior to the ventral disk and beat within the 'ventral groove'. The posterior lateral flagella emerge about two-thirds of the way back along the cell body.

Flagellar studies in *Chlamydomonas* (reviewed in Silflow and Lefebvre, 2001) and *Trypanosomes* (reviewed in Landfear and Ignatushchenko, 2001) have described the presence of several axoneme-associated structures within the cell body and localised several proteins important for flagella function and regulation to these structures. However, examination of the ultrastructure of these and other flagellates and ciliates reveals that while axoneme-associated structures are the rule, there is great diversity of morphologies and protein constituencies of these elements. The diverse structures associated with the proximal sections of axonemes within the cell body of *Giardia* are intriguing yet uncharacterised beyond their ultrastructure. Both the anterior and posterior lateral flagella are flanked on their posterior side by electron dense matter, termed the 'dense rods' (Filice, 1952) (Figs. 2C and 3A). Additionally the anterior axonemes have associated 'striated fibres' or 'marginal plates' running more along their anterior faces and extending into the tips of the ventrolateral flange (Fig. 3A) (Holberton, 1973a,b; Sogayar and Gregorio, 1991). The caudal flagella have associated dorsal and ventral short arrays of microtubules, the 'funis' of Kulda and Nohynkova (1978) and less substantive electron dense matter (Fig. 3B). The ventral flagella possess a fin-like structure of undetermined composition.

5.9. Ventral disk

The ventral disk of *Giardia* is a truly unique structure; even within the Diplomonads, only *Giardia* spp. have a ventral disk. The disk is a domed structure (Holberton, 1973a,b) that modulates concavity upon attachment to become more concave (Sousa et al., 2001). It is comprised of a clockwise (when viewed from the dorsal side; Ghosh et al., 2001) spiral array of microtubules emanating from a parallel set of undefined electron dense bands directly anterior to the ends of the caudal and posterior-lateral flagellar basal bodies (Fig. 3D). The ventral disk microtubules lie directly above the ventral plasma membrane and are attached by short fibres to it. Ribbon structures extend off the dorsal surface of each microtubule and are connected by a series of cross-links defined by Holberton and colleagues (Holberton, 1973a,b, 1981) (see enlarged view of disk in Fig. 3C). At the periphery of the disk, the microtubule and ribbon structures are replaced by a network of fibres in a region termed the lateral crest (Fig. 3D). It has been reported

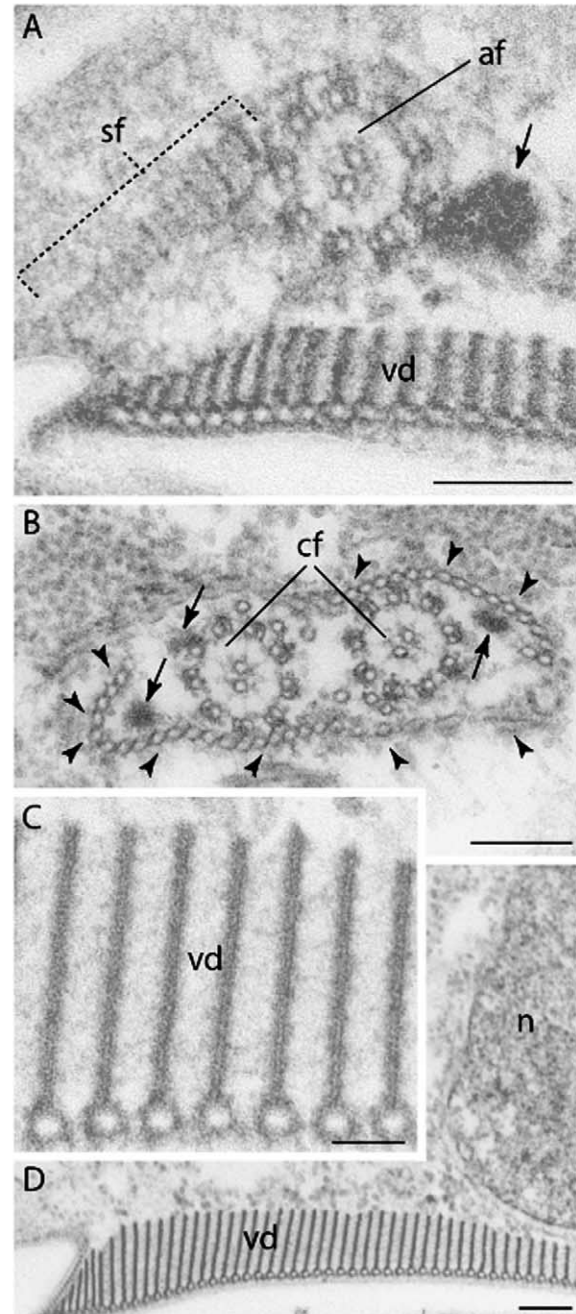

Fig. 3. Conventionally fixed TEM sections demonstrating structural components of typical *G. lamblia* trophozoite flagella/ventral disk. (A) High magnification view of anterior flagella (af) revealing the typical eukaryotic $9 + 2$ microtubule arrangement, along with the striated fibres (sf) and dense rod (arrow). (B) Transverse sectional view of caudal flagella (cf) and associated ventral/dorsal arrays of microtubules called the 'funis' (arrowheads). Dense rods (arrows) are also conspicuous. (C–D) Low and high magnification view of ventral disk (vd) demonstrating the typical parallel array of microtubules. Bars = 0.2 μm (A, B); 0.05 μm (C); and 0.1 μm (D).

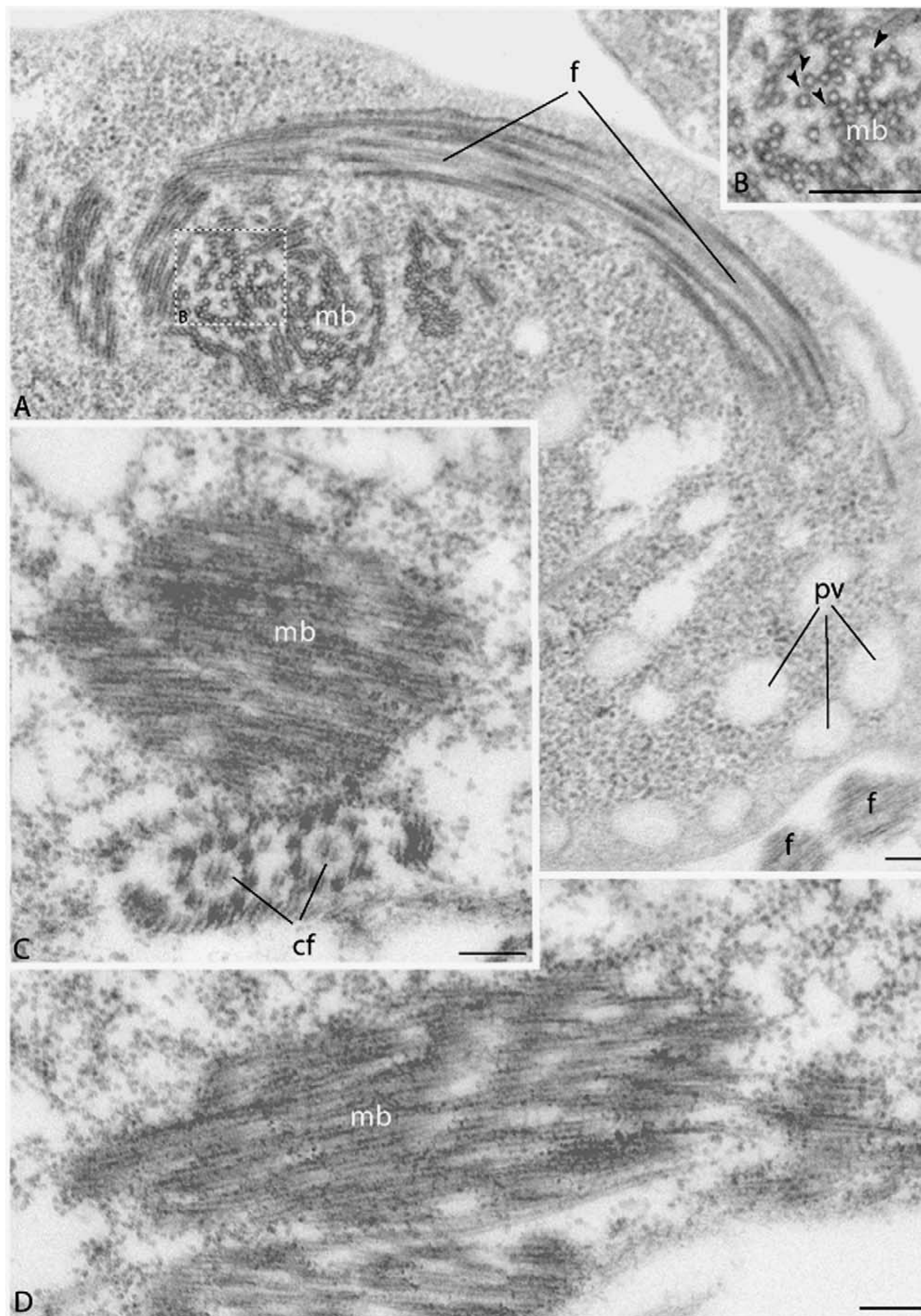

Fig. 4. Conventional TEM sections through *G. lamblia* trophozoites showing various presentations of the median body (mb). (A, B) Low and high magnification view of transversely sectioned median body. Note the perpendicular projections (arrowheads) extending from microtubules in B. (C, D) Various presentations of longitudinally sectioned medial body. Flagella (f); caudal flagella (cf); and peripheral vacuoles (pv). Bars = 0.2 μm.

that among the different species of *Giardia* the exact structure of the disk varies although it is unclear how much of the perceived difference is the result of different methods of structural preservation in different laboratories. In *G. lamblia* the centre of the disk is devoid of microtubules and ribbons in the 'bare zone' and the ends of the disk overlap. In some species, including *G. lamblia*, a notch is commonly observed at the posterior end of the disk.

5.10. Median body

The median body of *Giardia* is perhaps the least defined microtubule structure. Appearing in all species as a somewhat disordered array of microtubules posterior to the two nuclei, the function of the median body is unknown. The microtubules are roughly stacked lengthwise in a pile resembling a tumbled-down wood stack (Fig. 4). The exact shape

and position vary among the species; in *G. lamblia* the median body is elongated and tilted above the caudal flagella (Fig. 4B). Various presentations of the median body are seen in Fig. 4, with the transverse view shown in A–B; and the longitudinal view present in C–D. Note the lateral/perpendicular projections from the microtubules seen in panel B. These are of indeterminate origin.

6. Structural changes of the cytoskeleton during cell and developmental cycles

6.1. Cell division

Because of the basal phylogenetic position of *Giardia*, a more thorough understanding of mitotic mechanisms in this early diverging eukaryote could be relevant for comparative studies of mitotic evolution. Based on observations of both *Giardia* and *Hexamita*, a related diplomonad, nuclear division is presumed to be 'semi-open'. That is, the ends of the nuclear envelope associated with the centrosomes are fenestrated while the rest of the nuclear envelope is intact (Filice, 1952; Brugerolle, 1974; Nohynkova et al., 2000). Specific molecular and structural details of *Giardia* cell division, such as the assembly and function of the *Giardia* mitotic spindle and the proteins involved in the generation of force to segregate chromosomes, have not been investigated.

Two recent studies support two incongruent models of *Giardia* division, although both support the inheritance of a copy of each nucleus by the two daughter cells. Ghosh et al. (2001) contend that *Giardia* divide with ventral-ventral mirror image symmetry in the disc plane based on results from single nucleus labelling of episomal DNA by fluorescence in situ hybridization (FISH). Further, they argue that daughters are prevented from receiving two copies of the same nuclei by microtubules that tether the two mother nuclei and the two daughter nuclei. In contrast, Adam and coworkers using similar single nucleus staining (Yu et al., 2002) present evidence of ventral-dorsal division in which daughter parasites are stacked in the same orientation, as previously described by the work of Filice (1952). The discrepancy between the nuclear segregation data can be most easily explained by the attachment of fixed trophozoites to the slides in different orientations. While FISH is therefore suitable for determination of inheritance of nuclear content, it cannot stand on its own to provide direct evidence for the plane of cell division because the hybridisation buffer destroys cell morphology.

These alternative models for nuclear inheritance also impact models for replication and inheritance of the disk, flagella and median body, and it is through studies of these structures that we can gain an understanding of the pattern of cell division in *Giardia*. For example, the model of Ghosh et al. implies that the new disk forms on the ventral face of the old disk as a mirror image. However, as noted above, all disks spiral in the same orientation. It is also difficult to understand how a new disk assembles between the old disk and the ventral plasma membrane given that parasites remain attached until very late in cytokinesis (Nohynkova et al., 2000; and the common observation among researchers in the field of attached parasites with four nuclei). Cell division is a dynamic process, and GFP-labelled cytoskeleton proteins such as α- and β-giardin will be necessary to permit observation of cytoskeletal dynamics in living cells as they divide. Direct microscopic evidence of cytoskeleton rearrangements from fixed populations of dividing cells can also be obtained from indirect immunofluorescence using antibodies to α- and β-giardin and from electron microscopy studies.

The question of replication of cytoskeleton structures prior to cytokinesis is particularly interesting given the elaborate nature of the disk and flagella-associated structures. Two tantalising studies by Crossley and Holberton (1983, 1985) examined the ability of Sarkosyl-extracted cytoskeletons to reassemble in vitro. While a variety of filaments and sheets of various dimensions were formed, the precise microtubule array and dorsal ribbons of the ventral disk did not form. Given the complexity of in vitro reconstitutions, the best evidence for the stages of disk assembly will likely come from microscopy studies on intact cells as described above.

6.2. Encystment/excystment

The cyst stage of the *Giardia* life cycle is responsible for its spread to new hosts. Major cytoskeletal-mediated ultrastructural changes are known to occur during cyst morphogenesis. When *Giardia* changes from the half-pear trophozoite to the ovoid cyst, it then contains four nuclei with a replicated DNA content (Bernander et al., 2001). The formation of the cyst is marked by an expansion of the rough endoplasmic reticulum and Golgi deemed necessary to rapidly synthesise and secrete the two dominant cyst wall proteins, CWP1 and CWP2. The flagella and disk become irrelevant structures in the cyst, and one might expect that they would be depolymerised to monomers and stored until required to rebuild the cytoskeletal pieces during excystation. Intriguingly, *Giardia* is far more conservative in its approach, simply breaking the disk and flagella into several large fragments, storing these in the cytoplasm of the cyst, and apparently reassembling the fragments – like puzzle pieces – during excystation; the fragments are quite obvious in transmission electron micrographs of cysts (Sheffield and Bjorvat, 1977). The mechanism of fragmentation and reassembly is unclear, although various uncharacterised cytoskeletal motors could contribute to the mechanical force generation required for the subcellular reorganisation of the cytoskeleton and membranes during encystment and excystment. Yet despite the fact that most described morphogenic rearrangements are likely powered by the cytoskeleton, most work on encystation/excystation has

focused on the physiological signals responsible for encystment/excystment, protein trafficking, and the composition and development of the cyst wall (reviewed in Gillin et al., 1996; Lujan et al., 1997).

7. Proteins

As demonstrated in the previous section, the *Giardia* cytoskeleton is particularly rich in microtubules, as evidenced by their dominant presence in the ventral disk, flagella, and median body. Microfilaments have also been observed but appear to be much less abundant, and are most conspicuously seen in the cortical region of the cell. Research in the past decade on the *Giardia* cytoskeleton has focused on the identification of protein constituents. These can be roughly divided into several major categories: tubulin and associated proteins, actin and associated proteins, motor proteins, and various novel protein classes. Holberton and colleagues made the first important strides in this field through the two-dimensional gel analysis of detergent-extracted cytoskeletons and isolated flagella and formed estimates of at least 35 major proteins comprising the cytoskeleton (Holberton and Ward, 1981; Crossley and Holberton, 1983; Clark and Holberton, 1988).

7.1. Tubulins

Tubulin was first identified as a dominant protein constituent of *Giardia* by protein analysis of purified cytoskeletons (Holberton and Ward, 1981; Crossley and Holberton, 1983). Torian et al. (1984) then raised three monoclonal antibodies against *Giardia* lysate that reacted with 53 and 55 kDa proteins from *G. lamblia* and cross-reacted with purified bovine tubulin. Subsequent molecular analysis of promoter elements established that *Giardia* possesses two alpha-tubulin and three beta-tubulin genes (Kirk-Mason et al., 1988, 1989). Our analysis of the two α-tubulin genes determined that the two genes were 99.4% identical at the nucleotide level (five mismatches in 1,362 nucleotides) and 100% identical at the amino acid level (GenBank Accession numbers AY145881 and AF331826). Tubulin-based phylogenetic analysis is congruent with rRNA phylogenies in supporting the placement of *Giardia* within one of the earliest diverging eukaryotic lineages (Edlind et al., 1996; Keeling and Doolittle, 1996).

In better characterised eukaryotes, tubulin is subjected to a number of different post-translational modifications which, in part, appear to correlate with the distribution and stability of microtubules within cells although an assignment of cause and effect remains ambiguous (reviewed in MacRae, 1997; McKean et al., 2001). Investigation of tubulin modification in *Giardia* is of interest given the cell's highly stable microtubule-based structures (Holberton and Ward, 1981). Interestingly, the monoclonal antibodies against tubulin described above by Torian et al. were specific for either the flagella or the disk, although it is clear today that both structures contain tubulin. The nature of this segregated recognition has not been clarified, though it is possible that the monoclonal antibodies recognise different populations of modified tubulin. Indeed, Crossley and Holberton (1983) observed five different tubulin isoforms by staining of two-dimensional gels.

Most tubulin modifications occur near the C-terminus, including polyglycylation, polyglutamylation, phosphorylation, and a tyrosination/detyrosination cycle involving the terminal tyrosine residue (reviewed in MacRae, 1997; McKean et al., 2001).

Additionally, a highly conserved lysine at position 40 can be acetylated. Polyglycylation is seen only in very stable microtubule populations such as axonemes, and might be useful in dating microtubules; interaction with membranes may stimulate the polyglycylation process. Polyglutamylation is associated with microtubule formation; drugs such as nocodazole, which disrupt microtubules, inhibit polyglutamylation, while taxol, which stabilise microtubules, can enhance polyglutamylation. Although β-tubulin is more commonly phosphorylated than is α-tubulin, the functional significance of the modification is unclear. Detyrosinated and non-tyrosinatable tubulin are respectively associated with stable and very stable microtubule populations, although microtubule polymerisation is unaffected by tyrosination (reviewed in Idriss, 2000). The enzyme responsible for tyrosination, tubulin tyrosine ligase, requires a C-terminal glutamic acid to which to link the tyrosine, and a fuller sequence of Gly-Glu-Glu is strongly preferred. Like many of the other modifications, acetylation is associated with stable microtubule populations and can reduce drug- and cold-induced microtubule disassembly. However, as with the other tubulin modifications, acetylation correlates with, but does not cause, stability. Studies to significantly reduce acetylation in *Chlamydomonas* showed no phenotypic effect (Kozminski et al., 1993).

Not surprisingly, many studies have shown that *Giardia* tubulin is indeed highly modified to maximise stability. Indirect immunofluorescence and immunoelectron microscopy using monoclonal antibody 6-11B-1 specific for acetylated tubulin demonstrated that the full length of the axonemes, basal bodies, and median body were heavily labelled, while the disk was only lightly labelled (Soltys and Gupta, 1994). As further demonstrated by CNBr fragmentation and sequencing, α-tubulin appears almost fully acetylated at a conserved Lys-40 (Weber et al., 1997).

Analysis of *Giardia* α- and β-tubulin by HPLC and mass spectroscopy has also provided evidence for both polyglycylation and polyglutamylation (Weber et al., 1996, 1997). Polyglycylation is the dominant modification with ~40% of α-tubulin modified (Weber et al., 1996). In contrast polyglutamylation is less common, although a subset of the tubulin population is doubly modified (Weber et al., 1997). Polyglutamylated tubulin was localised by indirect immunofluorescence using GT 335 (a monoclonal antibody raised against a glutamylated peptide from α-tubulin). The

proximal halves of flagella were stained along with a faint nuclear fuzz; no staining of ventral disk or median body was observed (Boggild et al., 2002). Polyglycylated α- and β-tubulin was also detected using two monoclonal antibodies by Western blot analysis and localised by indirect immunofluorescence predominantly to the four flagella and median body, and more weakly to the ventral disk (Campanati et al., 1999).

The existence of a tyrosination/detyrosination cycle in *Giardia* has been the subject of debate. First, several lines of evidence indicate that *Giardia* α-tubulin is not tyrosinated. The C-terminal peptide sequence of Glu-Ala-Tyr argues against the ability of a tubulin tyrosine lygase to tyrosinate the protein, and tyrosinated tubulin could not be detected with two separate monoclonal antibodies to tyrosinated tubulin (TUB 1A2 from Sigma and YL1/2 from Accurate Chemical and Scientific) (Soltys and Gupta, 1994; Boggild et al., 2002). However, examination of C-terminal fragments of α-tubulin by carboxypeptidase Y digestion followed by mass spectrometry showed complete retention of the terminal tyrosine (Weber et al., 1997). Furthermore, a second group (Crossley et al., 1986) (again using the YL1/2 antibody) observed staining of the median body and, more faintly, staining of axonemes and ventral disk, despite rapid fading. Finally, our examination of the *Giardia* Genome Project (McArthur et al., 2000) reveals the presence of a gene (Contig 725, ORF 14498) with a high degree of similarity to other tubulin tyrosine ligase family genes, and the predicted protein sequence contains a canonical tubulin tyrosine ligase motif, suggesting that *Giardia* may indeed have a tyrosine ligase activity.

The robustness of the C-terminal sequence data showing complete C-terminal tyrosination of α-tubulin argues strongly against a tyrosination/detyrosination cycle in *Giardia*. This is unlike the situation in trypanosomatid flagella in which α-tubulin is rapidly detyrosinated (Sherwin et al., 1987; Sherwin and Gull, 1989; and reviewed in Kohl and Gull, 1998), but similar to the situation in yeast and some diatoms in which α-tubulin is fully tyrosinated (reviewed in Idriss, 2000). However, Weber and colleagues only examined α-tubulin assembled into the cytoskeleton, thus potentially missing an unpolymerised, detyrosinated form of α-tubulin and the presence of a tyrosination/detyrosination cycle, as our evidence for a tubulin tyrosine ligase gene family member would support. Although this explanation would still contradict a subset of the immunofluorescence data, that problem might lie in poor cross-reactivity of the antibodies to *Giardia* tyrosinated tubulin, much as we describe below for other heterologous antibodies. Indeed the 1A2 antibody was initially raised against the terminal 13 amino acids of tyrosinated mammalian α-tubulin (Kreis, 1987) which is not strictly conserved with *Giardia* α-tubulin. Resolution of this question will require protein sequence analysis of total α-tubulin content in *Giardia* – not just the assembled fraction – throughout the life and developmental cycles. Additionally, examination of *Giardia* cell lysates for tubulin tyrosine ligase activity should be performed.

Despite the tyrosine controversy and the absence of any data for phosphorylation, the clear evidence for polyglycylation, polyglutamylation and acetylation supports the idea that *Giardia* microtubules in the flagella and median body are exceptionally stable. The apparent lower levels of detectable tubulin modifications in the ventral disk may simply reflect limited accessibility of the disk to antibodies. Alternatively, the results may truly reflect lower levels of modified tubulin in the disk, which may, in turn, correlate with enhanced dynamics. Other cytoskeletal systems should also exhibit microtubule dynamics, however, no data are available for the extent and localisation of modified tubulin during periods of potential destability such as fission and encystation.

7.2. Microtubule organising centre proteins

Only two microtubule organising centre proteins have been identified in *Giardia*: gamma-tubulin and centrin. γ-tubulin is essential for the formation of functional mitotic spindles in protists, fungi and animal cells (Oakley, 1999). γ-tubulin has recently been identified in *Giardia* using monoclonal antibodies against the C-terminal peptide of human γ-tubulin (Nohynkova et al., 2000). γ-tubulin localises to four distinct basal body regions associated with the posterior-oriented flagella – likely the ventral and posterior-lateral pairs of flagella, although precise localisation still awaits analysis by immunoelectron microscopy. Localisation of γ-tubulin to the basal bodies is consistent with typical localisation of γ-tubulin in other cell types (Oakley, 1999). Because isolated median bodies and the bands localised at the point of origin for ventral disk microtubule array have been shown to nucleate microtubules in vitro (Feely et al., 1990), it is surprising that γ-tubulin staining was not observed at these locations. γ-tubulin staining of basal bodies disappeared during prophase, as is typical for mammalian cells but different from other flagellates and ciliates in which the staining remains throughout the cell cycle (reviewed in McKean et al., 2001). Even more unexpected was the lack of detectable γ-tubulin at the poles of mitotic spindles in dividing cells (Nohynkova et al., 2000). Although this may potentially reflect limitations of epitope detection, it is also possible that, despite the appearance of two spindle arrays, *Giardia* has a unique spindle pole composition and function. Given the limited understanding of mitosis in earlier-diverging eukaryotes, this is an important area for future study.

Centrin (also known as caltractin) is associated with basal bodies/centrioles/centrosomes/spindle fibres and functions in the duplication and segregation of centrosomes (reviewed in Salisbury, 1995). Important lessons concerning the role of centrin have come from *Chlamydomonas* in which centrin plays a role in microtubule (and flagella) severing. Centrin

was first identified in *Giardia* using a monoclonal antibody to algal centrin (Belhadri, 1995). Rod-like structures (flagella-associated) were observed between the nuclei and two spots at the anterior ends of the nuclei (coincident with basal bodies) also labeled. The gene for *Giardia* centrin was cloned (Meng et al., 1996) and localised using anti-human centrin antibodies showing the same localisation as above and also intense median body staining. To further characterise microtubule organising centres in *Giardia*, it will be necessary to localise centrin throughout the cell cycle; in particular the staining of the median body seen by Meng et al. (1996) is suggestive of a novel microtubule organising centre in *Giardia* and this observation deserves further exploration.

7.3. Giardins

Originally identified in the early 1980's by Holberton and colleagues, giardin was defined as a 29.4 kDa protein that associated with microtubules (Holberton and Ward, 1981; Crossley and Holberton, 1983), and purified fractions assemble into 2.5 nm filaments in vitro (Crossley and Holberton, 1985). The sequence which predicted a coiled-coil structure for this protein was identified in 1988 (Baker et al., 1988; Holberton et al., 1988) and came to be known as β-giardin. At this time, SDS-PAGE analysis of the cytoskeleton of *Giardia* revealed that at least two dominant proteins existed in the ~30 kDa range – termed α- and β-giardin; subsequent two-dimensional gel analysis revealed a far larger set of proteins (~23) in this size range (Peattie et al., 1989). Unfortunately because of their close proximity on protein gels, efforts to purify the two proteins and raise independent antibodies to the two were hampered by cross-contamination and apparent dominant antigenicity of β-giardin in α-giardin samples. Thus both proteins were eventually localised to the ventral disk (α-giardin incorrectly and β-giardin correctly) and, given the limited scope of the gene databases, thought to be unique to *Giardia*. This grouping of proteins was later found to be inappropriate as giardins have been shown to belong to at least three separate gene families: the annexin homologs – including α-giardins (Fiedler and Simons, 1995), the striated fibre (SF)-assemblin homolog – β-giardin (Weber et al., 1993), and the γ-giardins as proteins without notable homologs (Nohria et al., 1992).

Subsequent work with *Giardia* annexins has expanded the family and our understanding of their cellular roles. α1-giardin was characterised phylogenetically as annexin XIX, and α2-giardin as annexin XX (two new categories of annexins reflecting their divergence at the amino acid level) (Morgan and Fernandez, 1995; Morgan and Pilar-Fernandez, 1997) and further characterised functionally by their Ca2 + dependent binding to phosphatidyl serine vesicles (Bauer et al., 1999). Although α- and γ-giardin were initially localised to the ventral disk using rabbit polyclonal antisera raised against a 33 kDa protein band excised from a polyacrylamide gel (Peattie et al., 1989; Nohria et al., 1992), the antibodies were shown by the same authors to be cross-reactive with at least seven proteins. Subsequent localisation using a separate polyclonal antibody raised by a similar technique (Wenman et al., 1993) suggests that α-giardin is instead present at the dorsal plasma membrane. Data supporting a role for α-giardin in support of the dorsal membrane comes from C.C. Wang's laboratory: introduction of virus containing α-giardin DNA produced antisense transcripts within the cell that acted to profoundly distort not the ventral disk but the dorsal membrane (Köhler, S., Wang, C.C., 1996. *Giardia lamblia* is susceptible to α giardin antisense RNA. In: Molecular Parasitology Meeting VII. Woods Hole, MA). This membrane localisation is more consistent with the ability of α-giardin as an annexin to bind phospholipids (Bauer et al., 1999). A new annexin has recently been characterised as annexin XXI (Szkodowska et al., 2002). This protein shares the four common annexin helices and motifs and also binds to phospholipids in a Ca2 + dependent manner. Its localisation is unique, however, to the extracellular domains of the eight flagella, and more faintly to the median body. The authors propose the interesting and logical model that annexin XXI links the axoneme to the flagellar membrane.

The evolutionary relationship of β-giardin to SF-assemblin is intriguing. SF-assemblin is a structural protein from Chlamydomonas that is a part of 2.5 nm non-contractile filaments at the base of the flagella (Weber et al., 1993). Studies by Holberton and colleagues have shown that β-giardin also assembles into 2.5 nm filaments that then further assemble into the superstructures of the dorsal ribbons of the ventral disk (Holberton, 1981; Crossley and Holberton, 1985). Although the overall protein similarity is quite low (~20% identity and ~40% similarity), both proteins have similar coiled-coil domains and show a clear conservation of protein structure (Holberton et al., 1988). Recent experiments in *Chlamydomonas* involving SF-assemblin-GFP transfected cells and cells transfected with antisense constructs indicate that SF-assemblin is involved in flagellar assembly (Lechtreck et al., 2002). The localisation of β-giardin to the ribbons of the ventral disk has in contrast always suggested a primarily structural role for the protein. Thus, while the evolutionary relationship of SF-assemblin and β-giardin is clear, the functional relationship between the paracrystal rods of SF-assemblin and the significantly larger dorsal ribbons of β-giardin is unresolved.

Based on the aforementioned data, we argue for reclassifying the 'giardins' nomenclature to avoid prolonging any confusion regarding protein relationships. The α-giardins have already found acceptance in the literature as annexins, and references to α-giardins should be avoided in the future. The original giardin (β-giardin) should anchor the true giardin family. Because of a lack of homology to other protein families, proper classification and renaming of γ-giardin awaits a better understanding of the function of the protein.

7.4. Additional coiled-coil proteins

In addition to the smaller giardins, several other larger coiled-coil proteins have been identified in *Giardia*. Holberton and colleagues identified two coiled-coil proteins – a 101 kDa protein associated with the median body (Marshall and Holberton, 1993) and a 183 kDa protein (HPSR2) of undetermined cellular location (Marshall and Holberton, 1995). My laboratory has also recently identified a distinct 180 kDa protein (termed GASP-180) that shares with Holberton's 183 kDa protein the basic protein structure of a short (~200 amino acid) head domain, a hinge region, and a long coiled-coil tail (Elmendorf et al., unpublished data). In addition, the two ~180 kDa proteins also share consensus sequences for a P-loop motif in the head region raising the compelling possibility that they can bind to nucleotide triphosphates to promote binding/shape changes. The key difference between the proteins is the presence of a series of five ankyrin repeats in the head region of GASP-180.

As is typical for all coiled-coil proteins studied to date in *Giardia*, the coiled-coil regions of all three of these proteins do not fit the canonical coiled coil heptad repeat paradigm (Lupas, 1997). Preliminary analysis suggested that the coiled-coil domain would be frequently interrupted by skips and stammers (Marshall and Holberton, 1993, 1995). More recently it has been proposed that the coils assemble in unusual periodicity patterns (Hicks et al., 1997). These patterns incorporate both heptads (seven amino acids) and hendecads (11 amino acids) which have been synthesised as synthetic peptides and shown to assemble into coiled-coils in vitro. This increased flexibility in coiled-coil periodicity allows proteins to have a wider range of repeat patterns – e.g. 25 residues from a 7-11-7 pattern found in HPSR2 or 29 residues from an 11-7-11 pattern found in β-giardin, rather than the more constrained 21 residues resulting from a 7-7-7 pattern previously presumed standard for all coiled-coil proteins. Because these varied repeat patterns form coiled-coil domains with unique geometries, Hicks et al. (1997) further postulate that the function of this varied periodicity is to facilitate correct pairing of coiled-coil domains to ensure that the proteins form homodimers.

7.5. Actin and associated proteins

The family of actin and actin-related proteins is virtually unexplored in *Giardia*. The gene for *Giardia* actin was first identified in 1995, and the derived protein sequence is relatively divergent from other eukaryotic actin sequences with a 58% identity (Drouin et al., 1995). Southern hybridisation indicated that it is a single copy gene.

Polyclonal heterologous antibodies were used to localise actin and several actin-associated proteins, including alpha-actinin, tropomyosin, myosin and vinculin by indirect immunofluorescence assay (Feely et al., 1982; Narcisi et al., 1994). These antibodies, raised against chicken proteins, stained similar but not identical patterns at the periphery of the ventral disk and along the proximal end of a subset of the six posterior axonemes (Feely et al., 1982; Narcisi et al., 1994); the one exception was vinculin which localised predominantly to the entire ventral disk – including the bare zone (Narcisi et al., 1994). The localisation of actin and associated proteins to the periphery of the ventral disk has lent credence to the proposal that the lateral crest exhibits a contractile function in attachment (see below).

However, the limited localisation of actin to the periphery of the ventral disk raises several concerns about the heterologous antibodies used in these studies. First, we would expect actin to be present in additional locations within the cell – perhaps especially under the dorsal plasma membrane. The convex dorsal side of the parasite implies cytoskeletal support, and the absence of microtubules under the membrane points a finger at microfilaments. It is possible that an inability to detect all actin results either from protein divergence and the use of heterologous antibodies or is due to epitope inaccessibility. Second, similar reports of cross-reactivity between mammalian antibodies raised against cytoskeletal proteins, e.g. myosin, and yeast proteins turned out to be non-specific in most cases, and the antibodies did not recognise the expected proteins (H. Goodson, personal communication). Third, our analysis of the *Giardia* Genome Database (McArthur et al., 2000) has revealed no significant matches to alpha-actinin, vinculin, or tropomyosin genes (Elmendorf, Goodson and Dawson, unpublished data); we recognise that the sequence divergence noted for proteins in *Giardia* also raises the possibility that the protein identity is too low to be detected by simple Blast searches of the genome. The existence and function of actin-associated proteins remains a compelling research field in *Giardia*.

7.6. Motors

Motor proteins have been shown in other organisms to power the cytoskeletal movements behind cell division, vesicular transport, determination/maintenance of cell polarity, flagellar assembly/function, the functions of the ventral disc in attachment, and the morphogenic movements of the cytoskeleton during encystment/excystment. However, microtubule-based motor proteins, such as kinesins and dyneins, and the microfilament-based myosins, are virtually uncharacterised in *Giardia*. Segments of 13 different kinesin-related cDNAs were recently identified (Iwabe and Miyata, 2001). A phylogenetic analysis of four independently derived sequences placed them into three known categories of kinesins (KIN1 – a homolog to monomeric kinesins, typically involved in intracellular vesicular transport along microtubules; KIN2 – a homolog to BIMC kinesins and KIN3 – a homolog to MKLP kinesins, both typical components of mitotic spindles and an orphan kinesin, KIN4) (Lawrence et al., 2002). Dyneins are likely involved in flagellar assembly and function, mitosis, and intracellular trafficking, and although the *Giardia* Genome Database contains suggestive sequences (McArthur et al., 2000),

they have not been examined in *Giardia*. Furthermore, detailed analysis of flagella axonemes and microtubules in the median body reveals the presence of electron-dense hooks deriving from the peripheral array of nine doublet microtubules (Figs. 3B and 4A inset) that correspond morphologically to dynein arms in axonemes from other cell types. Similar arms have also been seen to protrude from the microtubules of the funis (Campanati et al., 2002).

Perhaps most intriguing is the apparent absence of any myosin gene in the *Giardia* genome. Although the previous studies examining actin-associated proteins also used heterologous polyclonal antibodies to localise myosin to the periphery of the ventral disk (Feely et al., 1982), these studies are subject to the same potential sources of artefact as described above. Again, in-depth and low-stringency analysis of the *Giardia* Genome Database (McArthur et al., 2000) has failed to reveal any myosin homologs (Elmendorf, Goodson and Dawson, unpublished data). It remains to be seen whether the myosin function is also absent or is accomplished by another unique protein in *Giardia*. While actin and its related proteins can obviously function in both structural and dynamic roles independent of actin (reviewed in Cramer and Mitchison, 1996), the complete absence of myosin in a eukaryotic cell is quite surprising and warrants further investigation.

8. Role in motility

Giardia's swimming behaviour has been noted since Leeuwenhoek commented that 'they made a quick motion with their paws, yet for all that they made slow progress' (Dobell, 1932). Two recent publications utilised video microscopy and low light imaging to investigate *Giardia* motility and provided more detail concerning the precise movement of the flagella (Ghosh et al., 2001; Campanati et al., 2002). However, our understanding of the specific actions of the flagella and the regulation of their differential beating patterns remains rudimentary. We include our own movies to highlight the various movements of the various pairs of flagella while *Giardia* remains attached to a glass substrate (Figs. 5A,B) and swims (Figs. 5C,D) to better illuminate the following descriptions.

Interestingly, the four pairs of flagella control different aspects of motility. The parasite has several distinct stages of motility: initial attachment to a substrate, prolonged attachment, detachment, and swimming behaviour. As described in more detail below, the ventral pair of flagella, which emanate from the posterior end of the disk area, beat continuously in a near sinusoidal motion in the plane of the disk while the parasite is attached (Holberton, 1973a,b; Ghosh et al., 2001; Campanati et al., 2002; Figs. 5A,B). The other flagella typically appear quiescent during attachment (Holberton, 1973a,b; Ghosh et al., 2001; Figs. 5A,B) although Campanati et al. (2002) and Erlandsen and Feely (1984) describe a rapid beating motion. An explanation for this discrepancy perhaps hinges upon the relative sensitivity of the flagella to microscopy conditions, but a final resolution awaits further analysis. Although the beating frequency of the various flagella pairs have been examined (Holberton, 1973a,b; Ghosh et al., 2001; Campanati et al., 2002), the broad range of measurements (10–18 Hz for the ventral flagella during attachment) likely reflects differences in cell activity within one preparation and also differences in experimental conditions (temperature, media, age of preparation) between experiments; therefore, absolute numbers are not practical.

The ventral flagella continue to beat when the trophozoites swim and are unequivocally joined by the action of the anterior flagella. Although the flagella appear to beat with similar frequencies, the motions are quite different, with the ventral flagella continuing the sinusoidal pattern and the anterior flagella moving asynchronously in bolder strokes (Holberton, 1973a,b; Erlandsen and Feely, 1984; Ghosh et al., 2001; Campanati et al., 2002: Figs. 5C,D). We observe a whip-like movement of the anterior flagella, with long undulations of the entire flagella that correspond temporally to forward motion of the parasite; thus, the parasite most resembles a swimmer doing the breaststroke (Figs. 5C,D). Although most researchers have found that the posterior lateral flagella beat while the parasite is swimming (Holberton, 1973a,b; Erlandsen and Feely, 1984; Ghosh et al., 2001; Figs. 5C,D), examination of recent video microscopy suggested to the researchers that the motion of the posterior-lateral pair of flagella might simply be due to waves generated by the ventral pair of flagella (Campanati et al., 2002). The caudal pair of flagella which emerge from the most posterior end of the cell body bend sharply during swimming behaviour, often guiding the course of the cell as a rudder steers a boat often causing the entire posterior end of the parasite to bend as well (Fig. 5D). The parasite can apparently also turn by varying the amplitude of the strokes taken by the anterior flagella (Figs. 5C,D). There is disagreement in the literature about whether beating of the caudal flagella contributes to trophozoite motility (Ghosh et al., 2001), although the general consensus argues against it (Holberton, 1974; Owen, 1980; Erlandsen and Feely, 1984; Campanati et al., 2002; Figs. 5C,D).

The resulting motion for the trophozoite is complex. The parasite moves forward with a 'jerk-and-glide' motion – primarily achieved by the beating of the anterior flagella. The parasite does not swim in a flat course, but rather rocks and rotates like a 'falling leaf' (Dobell, 1920). The rocking motion is assumed to be the result of the motion of the anterior pair of flagella as they beat in a slightly asynchronous pattern. Campanati et al. (2002) propose that the switch to a rotational motion is due to a change in direction of the wave action of the anterior flagella. Further examination of motility at the molecular level should emphasize flagellar function, regulation and assembly as each relates to parasite motility.

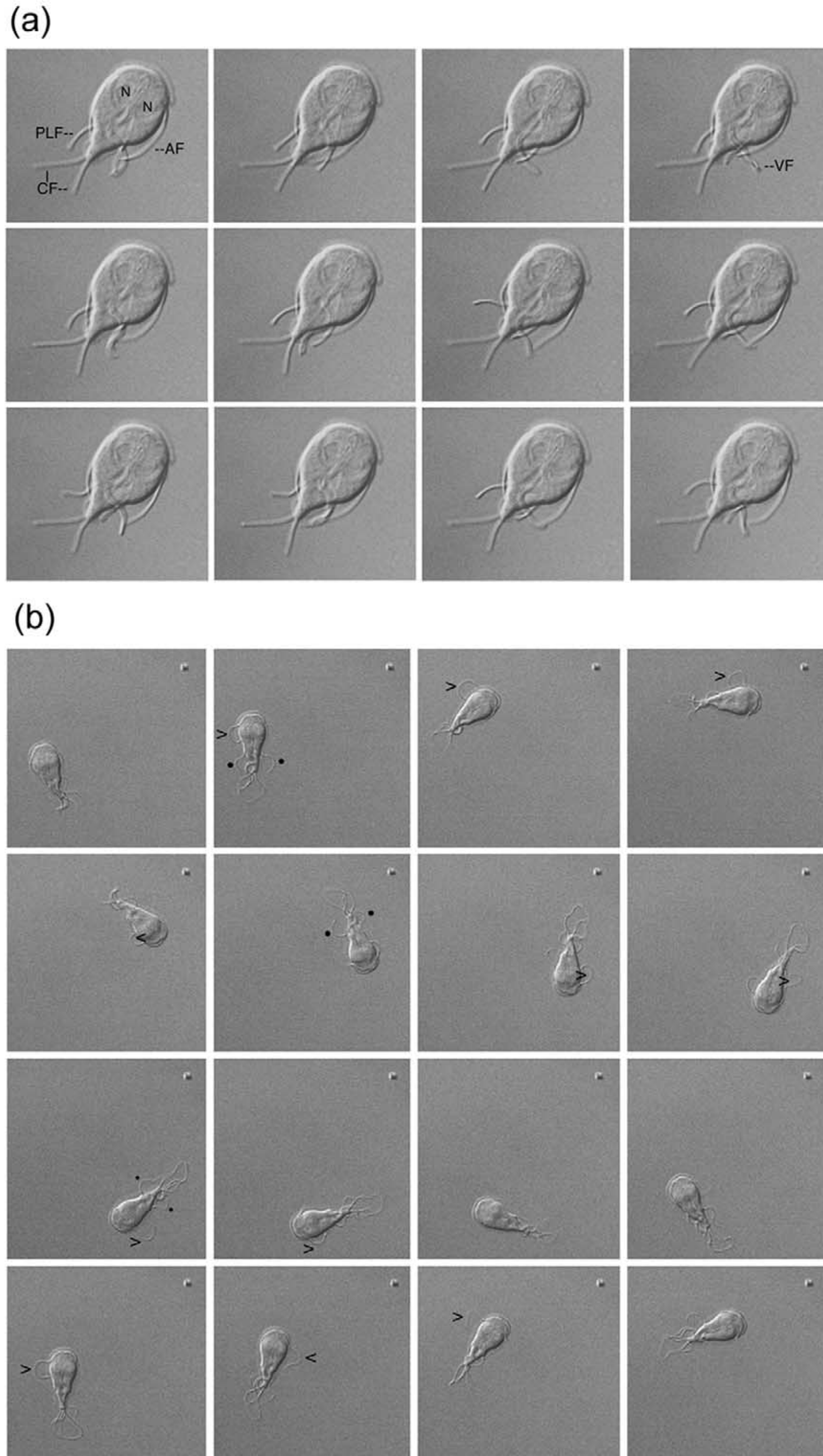

Fig. 5. Flagella motion in attached and motile *Giardia*. (A, B) The images are of attached trophozoites taken at ~0.1 s intervals. Panel A is focused in a plane slightly toward the dorsal side of an attached trophozoite and hence the nuclei are prominent. Panel B is focused in a plane slightly toward the ventral side of an attached trophozoite and hence the ventral disk is prominent. In both panels the ventral flagella can be seen to move in a clear sinusoidal wave pattern, while the posterior lateral flagella move only occasionally, and anterior flagella and caudal flagella do not appear to move at all. Abbreviations: N, nucleus; BZ, bare zone of disk; AF, anterior flagella; CF, caudal flagella; PLF, posterior lateral flagella; and VF, ventral flagella.

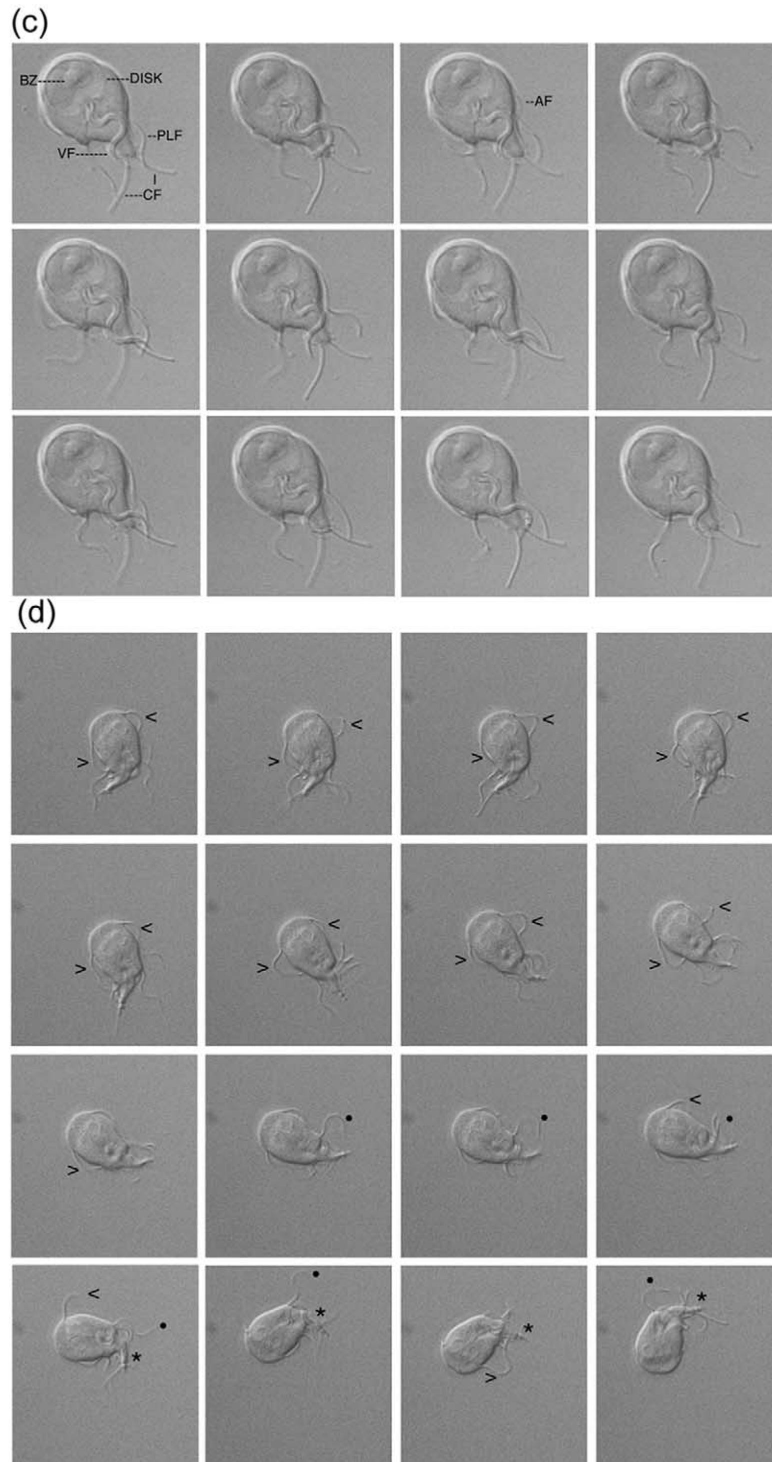

Fig. 5. (*continued*) (C, D) These images are of motile trophozoites. The trophozoite in panel C swims clockwise, while the trophozoite in panel D swims counter clockwise. It is unclear whether these are different rotation patterns or whether the trophozoites are in two different orientations (one dorsal side up and the other ventral side up). In both, the anterior flagella can be seen to take large whip-like strokes. Beating of the posterior lateral flagella is more prominent in the motile trophozoites, while the beating of the ventral flagella continues as in attached cells. The caudal flagella still do not appear to beat, although they are bent sharply in panel D. Turning of the trophozoites while they swim can apparently be accomplished both by flexing the posterior tail of the parasite and the caudal flagella as in panel D and also by uneven beating of the anterior and posterior lateral flagella as seen in both panels – the outer anterior flagella (the left in panel C and the right in panel D) beat in larger strokes than do the inner anterior flagella in panel D, especially, it is quite noticeable that forward motion of the parasite is accomplished by beating of the anterior and posterior lateral flagella (see the four panels in the bottom row). Symbols used: ' > ', left anterior flagella; ' < ', right anterior flagella; '●', posterior lateral flagella; and '*', tail and caudal flagella. Images were taken on Zeiss Axiophot under 63 × or 100 × magnification and acquired using a Photometrics CoolSnapFX camera using OpenLab software from Improvision, Inc. Images were further processed using Adobe Photoshop.

9. Role in detachment

Giardia must be able to swim and attach to the host intestinal epithelial layer to avoid being evacuated by peristalsis. However, because intestinal cells and mucous layers are continually shed, it is equally imperative that the trophozoite be able to detach from an unsuitable surface and swim and attach to a better one. This continual cycle of swimming, attachment, and detachment is readily observed in vitro, yet the processes of attachment and detachment have not been explored. Recent video microscopy experiments by Campanati et al. (2002) suggest a role for the ventral disk in detachment – apparently by contraction of either the anterior or posterior end of the disk – thus freeing the parasite and permitting it to swim. Earlier, Erlandsen and colleagues had shown by time lapse imaging and electron microscopy that detachment is often directly preceded by flexion of the posterior end of the parasite sharply upward (Erlandsen and Feely, 1984). Although earlier observations concerning the caudal flagella predicted that the orientation of the microtubules within the axoneme would preclude beating in a motion to facilitate this flexion, more recent observations suggest that the caudal flagella may indeed beat in a ventral-dorsal motion conducive to tail flexion (Ghosh et al., 2001). It is unclear whether disk contraction is independent of, or dependent upon, the dorsal flexion.

10. Role in attachment

Giardia attaches to epithelial cells to maintain infection within the host's small intestine and to prevent elimination by peristalsis. At the basic level – attachment is contingent on temperature, pH, and ionic strength, with optimal attachment occurring at physiological conditions (Gillin and Reiner, 1982). *Giardia* must be biologically active to attach; fixation with glutaraldehyde (McCabe et al., 1991; Sousa et al., 2001), chilling to 4°C (Gillin and Reiner, 1982), and treatment with iodoacetate to halt glycolysis (Feely and Erlandsen, 1982) all dramatically block attachment. *Giardia* attaches to both biological and inert substrates, including a wide range of mammalian cell lines, plastic and glass.

Five separate models of attachment have been proposed in the literature, and although arguments exist both for and against each model, the experimental evidence is not conclusive. (1) Several groups propose a lectin/sugar interaction, noting that the surface of *Giardia* is negatively charged (Gonzalez-Robles et al., 1989), and have further attributed this charge to the presence of *N*-acetyl-D-glucosamine residues on the parasite surface (Farthing et al., 1986; Ward et al., 1988). (2) The beating action of the ventral pair of flagella during attachment generates a negative pressure under the disk that attaches the cell to the substrate by a mechanism similar to a suction cup (Soloviev, 1968; Holberton, 1974). (3) The spiral array of microtubules in the ventral disk contracts by tightening or loosening the spiral array; this in turn alters the disk diameter and achieves attachment by a clutching action (Mueller et al., 1974). (4) The detection of actin at the periphery of the ventral disk provides support for the lateral crest as a contractile region. In this model the spiral array of microtubules need not alter its lateral configuration, but instead is buckled upward and the disk achieves a more pronounced concave shape. Attachment is again achieved by a clutching action (Friend, 1966; Sousa et al., 2001). (5) The observation in many transmission and scanning electron micrographs of a tight attachment between the periphery of the ventrolateral flange and host epithelial cells has suggested to some that the ventrolateral flange region participates in attachment (Friend, 1966; Sousa et al., 2001). We will briefly review the available research examining possible roles for lectins, flagella beating, and disk dynamics in attachment, with a special emphasis on studies using drugs believed to target the cytoskeleton. Although the field is still contentious with no definitive answers, a number of well-documented studies support several of the models, and it is most plausible now to postulate that while lectins likely serve a secondary role in recognition of proper cell substrates in the small intestine, the contraction of the periphery of the lateral crest to dig into microvilli of the epithelial cells is the dominant force in attachment.

10.1. The role of lectins

A role for lectins in attachment of *Giardia* to host cells was originally proposed by extrapolation from other intestinal parasitic protists (e.g. *Entamoeba histolytica* (reviewed in Gilchrist and Petri, 1999)) and was first supported by evidence that treatment of parasites with high levels of trypsin (0.5–1.0 mg/ml) prior to incubation with epithelial cells reversibly prevented attachment (Inge et al., 1988; Pegado and de Souza, 1994). Furthermore, 100 mM periodate treatment of *Giardia* parasites (but not host cells) to block hydroxyl groups on sugars decreased attachment by ~60%. It has also been observed that *Giardia*, although capable of attachment to inert substances, attaches with variable affinity to different intestinal cell lines thus implying a specificity of interaction missing from a purely mechanical explanation (Inge et al., 1988). Attempts to determine the exact nature of the lectin interaction has proven more complicated with (sometimes contradictory) evidence supporting a role for glucose, fucose, galactose, mannose, mannose-6-phosphate, α-D-methylmannoside, *N*-acetyl-glucosamine, and *N*-acetyl-galactosamine in blocking of attachment (Inge et al., 1988; Magne et al., 1991; Pegado and de Souza, 1994; Katelaris et al., 1995). Additionally, pretreatment of parasites with many different lectins (with the best evidence provided for wheat germ agglutinin) decreases attachment (Magne et al., 1991; Pegado and de Souza, 1994).

While these results implicate sugars in the mediation of specific interactions between *Giardia* and host cells, it is

clear that dynamic actions of the cytoskeleton are sufficient for attachment. This conclusion is supported by the basic observation that *Giardia* is highly capable of attachment to glass, plastic surfaces and a wide range of mammalian tissue culture cell lines, including small intestine, large intestine and kidney cell lines. A multitude of electron micrographs also clearly demonstrates the interdigitation of the edges of the disk (crest) with microvilli. Thus, it is the role of the cytoskeleton in attachment upon which most of the research effort has focused.

10.2. The hydrodynamic model and the role of the ventral flagella

Soloviev and Holberton's independent observations in the 1960's and 1970's of the ventral disk structure and the continued beating of the ventral pair of flagella during attachment led them to a hydrodynamic model of attachment in which beating of the ventral pair of flagella generates a fluid flow and consequent negative pressure beneath the cell – a suction force to keep the cell attached (Soloviev, 1968; Holberton, 1973a,b, 1974). It should be noted that this model also assumes an upward arching of the disk to allow *Giardia* to grasp into the microvilli (Holberton, 1973a,b, 1974).

An acknowledged problem with this explanation is that it cannot account for parasite *detachment* since the ventral pair of flagella apparently beat continuously, regardless of the parasite's actions. More fundamentally, however, Holberton's biophysical calculations relied on a model of trophozoite morphology that is now generally accepted as incorrect: he had postulated that the ventral flagella emerged from the centre of the disk, when they have subsequently been shown to emerge from the cell body in the ventral groove dorsal to the disk (Feely et al., 1984). Later work proposed that the negative pressure is generated in the marginal groove, the space between the ventrolateral flange of the cell body and the lateral crest of the disk. Indirect evidence for this comes from experiments by Ghosh et al. (2001) in which they report the intake and flow of beads around the periphery of the ventral disk. These experiments raise the interesting possibility that the beating of the ventral flagella and the concomitant negative pressure may act to sweep matter under the parasite, perhaps for the purpose of feeding. Further potential problems stem from observations that although fixation of swimming, non-attached parasite prevents attachment (McCabe et al., 1991; Sousa et al., 2001), fixation after attachment (and thus immobilisation of the flagella) does not force detachment (Erlandsen and Chase, 1974; Mueller et al., 1974; Owen et al., 1979; Feely and Erlandsen, 1981, 1982). This raises questions about whether the ventral flagella beating is indeed essential for attachment. However, post-attachment fixation likely also cross-links the parasite to the substrate or cells used for the attachment assay, which might prevent natural detachment; thus, a true understanding of the role of the ventral flagella in attachment is still lacking.

10.3. Disk dynamics

Detailed structural analysis of the disk in the 1970's and 1980's by Holberton's laboratory (Holberton, 1973a,b, 1974) indicates that the dorsal ribbons of the disk are linked with filaments of an undefined protein composition (Holberton, 1981). These linkages would need to be broken to permit the disk to expand and contract like a coil. Thus, the early proposal that the spiral array of microtubules itself tightens to change the disk diameter and attach the parasite seems unlikely given our current understanding of ventral disk structure.

Evidence for a role for the lateral crest in attachment is intriguing, but unfortunately only circumstantial. Direct contact of lateral crest with epithelial cells has been repeatedly observed (e.g. Erlandsen and Feely, 1984), showing a pronounced contraction of the edges of the disk that results in pictures of the parasite seemingly grasping the microvilli of epithelial cells much as a cat's claw hooks into carpeting. This contact is supported by interference-reflexion images as well (Erlandsen and Feely, 1984). The effects of parasite attachment seem limited to a shortening and thickening of the microvilli in a limited number of attached cells ($<10\%$) (Chavez et al., 1986) with the greatest distortions seen in the lateral crest zone of attachment (Koudela, 1994). Scanning electron micrographs from several studies of epithelial cells in infected animals have revealed the presence of 'footprints' left behind by detached parasites (Erlandsen and Chase, 1974; Chavez et al., 1986; Sousa et al., 2001). These 'footprints' show a sharp mark corresponding to contact with the lateral crest region of the ventral disk and a fainter impression of the spiral pattern of the ventral disk.

In transmission electron micrographs of attached cells, the ventrolateral flange is often shown tightly opposed to the epithelial cell layer. Interference-reflexion microscopy images also clearly show attachment by not only the lateral crest as noted above, but also the ventrolateral flange (Erlandsen and Feely, 1984), and scanning electron micrographs of epithelial cells post-attachment also show a rim of microvilli removal. Sometimes a wider spread of destruction is seen, perhaps reflecting the ventrolateral flange attachment as well (Koudela, 1994).

The observation of actin localising to the periphery of the disk lends credence to the idea that the fibrous matter in the lateral crest can contract. Interestingly, Chavez and Martinez-Palomo (1995) have further noted that the lateral crest region is relatively devoid of intramembrane proteins and cholesterol complexes which they propose may relate to the crest's contractile properties. A direct test of this model has not been done, although numerous drug studies described below have worked to investigate the topic indirectly.

10.4. Studies with drugs to target the cytoskeleton

Numerous studies within the past decade have examined the effect of various chemicals on attachment. Several factors confound interpretation and comparison of these studies: laboratories have used different strains (and species) of *Giardia*, different substrates and cell lines for attachment, and conducted the attachment assays in different media. When results are contradictory, it is therefore difficult to discern the underlying cause of the discrepancy. Furthermore, although the majority of the effort has focused on drugs proposed to target cytoskeleton structures, data concerning the effectiveness of cytoskeleton drugs, particularly colchicine, in other protists (reviewed in Gull, 1999) call into question our ability to extrapolate drug efficacy on *Giardia* proteins. Most importantly, the drug-treatment literature in *Giardia* typically does not examine either cell viability or cytoskeleton ultrastructure when examining either the specificity or effect of the drugs on their proposed targets. We therefore summarise the data as they are described in the literature but refrain from drawing conclusions from the results of specific experiments.

The results from these various experiments are summarised in Tables 1 and 2. We have grouped the drugs into two classes that are presumed to impinge either on microtubule structure (colchicine and nocodazole) or on microfilament structure (cytochalasins). The actions of a third class of drug, the benzimidazoles, are discussed separately below. The addition of colchicine or nocodazole to parasites prior to incubation with epithelial cells seemed to have only a minimal effect on attachment, while addition of the drugs during the incubation of parasites with epithelial cells diminished the ability of the parasites to attach. Taken in aggregate, these results are consistent with one of three conclusions: (1) host microtubules, but not parasite microtubules, are necessary for attachment; (2) drug effects on parasite microtubules are rapidly reversible and are eventually ineffective over the course of 1–2 h attachment assay; or (3) neither colchicine nor nocodazole is effective against parasite microtubules. Treatment with cytochalasins gives rise to more varied results – from profound inhibition of attachment to enhanced attachment. However, the majority of experiments suggest that cytochalasins (regardless of type) decrease attachment, implicating a role for microfilaments in attachment.

A series of papers have reported the results of experiments with benzimidazoles on attachment and, importantly, have also assessed the drugs' effects on parasite morphology and ultrastructure. Several benzimidazoles are highly effective anti-giardiasis drugs given to livestock, and there is significant interest in understanding their mode of action against *Giardia*. In worms and fungi, the benzimidazoles have been identified as effective disruptors of microtubules through their ability to bind to β-tubulin and through the resistance of various β-tubulin mutants to benzimidazole treatment (Roose, 1990; Kwa et al., 1994; Lacey and Gill, 1994). The effects of albendazole and mebendazole on *Giardia* attachment have been studied in detail. Several studies have confirmed a profound impairment of attachment by <1 μg/ml mebendazole with significant distortion of parasite morphology noted in several papers and described further below (Edlind et al., 1990; Chavez et al., 1992a,b; Oxberry et al., 1994, 2000; Sousa et al., 2001). Albendazole was examined in one paper that found it even more effective against attachment (Edlind et al., 1990). The linkage of cytoskeleton and attachment to drug efficacy seems to be specific to the benzimidazoles based on comparative studies noting the absence of an effect of metronidazole on attachment despite the ability of metronidazole to kill *Giardia* (Feely and Erlandsen, 1982; Katelaris et al., 1994).

Evidence from ultrastructural observations of benzimidazole-treated cells implicates the microtubular cytoskeleton as a drug target (Oxberry et al., 1994). Over the course of 32 h of treatment with 10 μM albendazole, microtubules and microribbons appeared to scatter and the cells to swell. This same effect was seen in untreated cells left in culture after stationary phase, leading the authors to suggest that albendazole mimicked the effect of natural cell death (Oxberry et al., 1994). Efforts to localise sites of albendazole and albendazole metabolites within the trophozoite showed scattered staining in the cytoplasm of the cell, although eventual localisation of albendazole sulphone could be seen above the median body (Oxberry et al., 2000). Treatment of trophozoites with albendazole at concentrations of 0.1 μg/ml resulted in disastrous structural effects with fracturing of the ventral disk, although axonemes appeared intact and the overlying ventrolateral flange was not distorted (Chavez et al., 1992a,b).

Within the array of experiments described above, there are several experiments that have moved beyond observation to test the various models of attachment previously outlined. Although we are still far from understanding the complex synergy by which factors regulate the attachment of *Giardia* to substrate, these experiments provide revealing insights into the mechanism of attachment. The hydrodynamic model of attachment has been recently supported in the literature by the observations of Ghosh et al. (2001). They report (but unfortunately do not show any data for) an experiment in which they observe the intake and flow of beads around the ventral disk as predicted by current revisions of the hydrodynamic model with negative pressure generated between ventrolateral flange and lateral crest. Alternatively, Campanati et al. (2002) investigate a role for beating of the flagella – as creators of the negative pressure – in attachment. The addition of high levels of Percoll (60–90%) to slides of attached *Giardia* virtually stopped the flagella beating prior to cell death; however, despite the absence of beating, the cells did not detach from the glass slides (Campanati et al., 2002). Thus, although the continued beating of the ventral flagella has always been assumed to indicate a need (otherwise why should *Giardia* waste the

Table 1
Effect of drugs presumed to target microtubules

Drug	Concentration	Drug application	Effect on attachment	Substrate and media	Attachment assay	Ref.
Colchicine	10 nM–100 μM	Trophozoites treated during 60 min time course	No significant effect	Polystyrene in HBSS	Cell counts by microscopy	Feely and Erlandsen (1982)
	100 μM	Trophozoites-pretreated for 30 min	No significant effect	IEC-6 in DMEM	[^{3}H]thymidine incorporation and trypan blue viability assay	McCabe et al. (1991)
	10–50 mM	During 60 min co-incubation	Decrease by 35%	Caco-2 in MHSP3	Counted in hemacytometer	Magne et al. (1991)
	12.5–125 μM	During co-incubation	Decrease by 35%	Caco-2 in MHSP3	Counted in hemacytometer	Katelaris et al. (1995)
	2.5–5.0 mM	During 2 h co-incubation	Decrease by >50%	INt-407 in RPMI	Counted in hemacytometer	Sousa et al. (2002)
Nocodazole	10–50 mM	During 60 min co-incubation	Decrease by 72%	Caco-2 in MHSP3	Counted in hemacytometer	Magne et al. (1991)

Table 2
Effect of drugs presumed to target microfilaments

Drug	Concentration (μg/ml)	Drug application	Effect on attachment	Substrate and media	Attachment assay	Ref.
Cytochalasins (B–D)	10–25	Trophozoites treated during 2 h time course	No significant effect	Glass in MM-2	Counted in coulter counter	Gillin and Reiner (1982)
(B)	10	Trophozoites treated during 60 min time course	Decrease by 40%	Polystyrene in HBSS	Counted by microscopy	Feely and Erlandsen (1982)
(B)	10	Trophozoites pretreated for 30 min	No significant effect	Fresh rat enterocytes in HSCB	Counted by phase microscopy	Inge et al. (1988)
(A, B and D)	5–20	Trophozoites pretreated for 30 min	Decrease by 50–85%	IEC-6 in DMEM	[^{3}H]thymidine incorporation and trypan blue viability assay	McCabe et al. (1991)
(B and D)	20–50	Trophozoites treated during 60 min co-incubation	Increase by 20–24%	Caco-2 in MHSP3	Counted by hemacytometer	Magne et al. (1991)
(B)	1–20	Trophozoites pretreated for 15 min; no drug present during co-incubation	Decrease by 34%	Caco-2 in MHSP3	Counted by hemacytometer	Katelaris et al. (1995)
(B and D)	5–10	Trophozoites pretreated for 30 min; no drug present during co-incubation	No significant effect	Int-407 in RPMI	Counted by hemacytometer	Sousa et al. (2002)
(B and D)	2.5–5	Epithelial cells pretreated for 60 min; no drug present during co-incubation	Decrease by 10–20%	Int-407 in RPMI	Counted by hemacytometer	Sousa et al. (2002)

energy?), this experiment suggests that the flagella may not be necessary for attachment. Interpretation of the data is unfortunately confounded by the imminent death of the cells. Date from Oxberry et al. (1994, 2000) indicates that an intact ventral disk is important, although the function of the disk (roof of a negative pressure chamber? grasping structure?) is not investigated. By investigating the effects of albendazole, one of the most potent inhibitors of attachment, they found that flagellar axonemes of treated cells remain intact while the disk and general cell morphology is profoundly distorted. While determining the drug target is clearly important, the broad effects of treatment preclude a precise commentary on structure-function relationships.

11. Future directions

As this review has described, our current knowledge of the *Giardia* cytoskeleton is limited primarily to the structural and observational levels. We have a detailed analysis of the major structural components of the cytoskeleton, but only a limited knowledge of its basic protein constituents of the cytoskeleton. Moreover, our understanding of the function of these structures and proteins is even more limited, and insights into the regulation of the dynamic processes are almost completely lacking. We therefore describe below important avenues for future cytoskeletal research, focusing on each of these three major areas: structure, function, and regulation.

11.1. Understanding protein composition of the cytoskeleton

Protein identification will be facilitated by the newly available tools in genomics and proteomics combined with previously developed biochemical and microscopy techniques. The availability of the genome project as it nears completion permits a comparative genomics approach to the cytoskeleton of *Giardia*. The cytoskeletal components and genomes of the later-diverging eukaryotes such as budding yeast and *Caenorhabditis elegans* are well-studied. Thus, the search to characterise the proteins in *Giardia* can receive a jumpstart through an analysis of the genome and a search for homologs of eukaryotic cytoskeleton proteins. While this approach will clearly have its limitations, especially considering the extreme divergence of many *Giardia* proteins to their homologs in other cells, it is the most rapid means to identify putative (and testable) homologs. In particular, the paucity of knowledge concerning microtubule-associated proteins, actin-related proteins, and motor proteins is limiting research effectiveness in *Giardia*.

The genome project also facilitates a proteomics approach to the identification of protein components of specific cytoskeletal components. Holberton's development of cytoskeleton and flagella purification protocols enabled him to analyse their protein composition by two-dimensional gel analysis (Crossley and Holberton, 1983, 1988). While this work revealed the wealth of different proteins that comprise the various cytoskeletal elements, identification of the proteins was hampered by a lack of specific tools. Protein 'spots' on two-dimensional gels can now be identified either by the development of monoclonal antibodies raised against specific cytoskeletal fractions, akin to the work of Woods et al. (1989) in *Trypanosomes*. More directly, however, the availability of the genome permits protein identification by mass spectrometry analysis of the electrophoretically-isolated proteins followed by extrapolation to the full protein sequence by localisation of the peptide sequence within the genome. This approach will prove particularly important when examining novel protein families in *Giardia* – a point at which comparative genomics approaches typically fail. These novel sequences will also provide key insights into the diversity of the eukaryotic cytoskeleton and pinpoint possible drug targets.

The presence of novel structures, both large (the disk) and small (the multitude of dense fibrous rods), have long puzzled cytologists and, as described in this review, our understanding of them has long remained at the purely descriptive level. To proceed, we therefore need to first link our new knowledge of protein identification with specific cytoskeletal structures and then subsequently with function. Proteins can be localised to structures within intact cells or cell fractions using carefully defined reagents (monospecific antibodies, GFP- or epitope-tagged constructs) at the light and ultrastructural level. The advent of molecular tools should also not eclipse the value of more traditional approaches to protein function. Protein networks that comprise a structure have yet to be investigated by standard protein purification and interaction methodologies such as co-immunoprecipitations.

11.2. Functional analysis of the cytoskeleton

Once the key constituents of the *Giardia* cytoskeleton (or at least of key structures) are defined, studies can be designed to illuminate structure-function relationships. These studies will rely heavily on observations of dynamics in living cells. The advent of video imaging has provided researchers with the ability to observe and capture *Giardia* motility behaviour; in particular low-light imaging permits visualisation of cells without the harmful effects of high light intensity that have plagued researchers previously. The ability to introduce GFP-tagged constructs further permits visualisation of the dynamics of individual proteins in living cells (Hehl et al., 2000; Elmendorf et al., 2001). The ability to follow real-time movements of proteins is likely to prove particularly illuminating in studies of cell division and the encystation/excystation cycle. Because of the difficulty in precisely synchronising *Giardia* cultures, these are processes that have previously been seen only as isolated snap shots, with the progression of events difficult to capture and study. A specific example of a compelling question revolves around disk assembly. No evidence exists for how the disk replicates

during cell division, and the continual appearance of large disk fragments in cysts provokes the tantalising assumption that the disk simply reassembles during excystation, rather than undergoes de novo synthesis. Examination of disk dynamics with GFP-tubulin or β-giardin-GFP will permit a careful analysis of these processes.

Although the dynamic viewing of cellular processes through the movements of a single protein will improve our understanding of the role that the protein plays in the life of *Giardia*, a more precise understanding of a protein's function can best be obtained from the study of mutant cell lines in which the protein is depleted. Antisense (and hopefully also RNAi) can be used to reduce protein levels and study of the resulting phenotype will link protein and function. Similar studies can be performed to study individual protein domains through the generation of dominant-negative mutants. One example of the power of this approach would be to examine flagellar function. The discovery of proteins localised exclusively to flagellar-associated structures specific for a given pair of flagella could be followed with studies in which the levels of that protein are depleted. Examination of the flagellar-associated ultrastructure and any effects on trophozoite motility or attachment would provide precise insight into the function of a pair of flagella. This level of analysis is not available through the use of drugs since they would typically target not only all of the flagella, but also a large subset of cellular microtubules.

11.3. Regulation of cytoskeleton function

A full knowledge of protein composition and function of cytoskeletal structures assumes a steady-state of cell organisation and function. In reality, of course, *Giardia* is continually interpreting environmental signals to effect changes in cytoskeletal structure. and function. For example, how does *Giardia* differentially control the actions of its four pairs of flagella? How is cell attachment/detachment triggered?

Unfortunately, our current understanding of possible regulation of cytoskeleton function is limited to a recent study by Chakrabarti and colleagues which identified a protein kinase A in *G. lamblia* (Abel et al., 2001). This protein localises to the flagellar basal bodies and along the intracellular axonemes of the pairs of anterior and posterior-facing flagella (tentatively identified at the light microscopy level as the caudal flagella by the authors). Although the basal body localisation was constitutive, the axoneme localisation was more evident at lower cell densities, after feeding of the cultures, and at higher cAMP levels. The authors also noted a need for protein kinase A activity during early stages of excystation. The localisation of protein kinase A to the basal bodies and its requirement in excystation suggest a role in organisation or assembly of axonemes. Furthermore, the localisation of protein kinase A to a subset of flagellar axonemes permits speculation that it may play a role in differential control of flagella beating. And in turn, the sensitivity of protein kinase A localisation to classic cell signals such as cAMP and cell density implies a means by which *Giardia* might translate environmental conditions to changes in behaviour. Chakrabarti and colleagues have provided the first glimpse into the regulation of the cytoskeleton in *Giardia*.

An area of research ripe for analysis of regulatory pathways is cell attachment. Although significant research effort has been devoted to the attachment of *Giardia* to host cells, in reality we are not much closer to an answer today than we were back in 1974 when Holberton first deeply probed the mechanism of attachment. Additional drug studies need to be performed with examination of the effect on both trophozoite behaviour and ultrastructural morphology to permit structure-function correlations. Even then, however, perhaps the most important question from an interventional perspective will remain unanswered – how does *Giardia* attach at the proper time and place? If prior experiments point to a role for specific proteins, our questions can rapidly become more focused and examine single protein behaviour. Biochemical studies can be performed to examine whether protein interactions are affected by intracellular conditions (e.g. the effect of cAMP levels as described above). Searching for targets of protein kinase A and other cytoskeleton-associated kinases may also point the way to regulated proteins.

12. Conclusions

The prior discussion demonstrates the plethora of fundamental, yet approachable questions concerning the structure and function of the cytoskeleton of *G. lamblia*. The direct relationship among attachment, motility and the cytoskeleton clearly has far reaching implications in understanding the basis of disease and thus demands further research. From an evolutionary standpoint, the extreme divergence of *Giardia* and the resulting novelty of structures and associated proteins suggests that we still have much to learn about cytoskeletal evolution and organisation in eukaryotic cells.

The ability to readily culture *Giardia* and complete the life cycle in vitro make *Giardia* approachable for biochemical and microscopy studies of cells and flagella. More recently, the development of molecular tools for stable transfection and expression of GFP-tagged proteins and the ability to perform 'knock-down' experiments to reduce protein levels and permit cell manipulation will allow researchers to delve more deeply into functional, rather than strictly observational, studies. The *Giardia* genome project adds the final missing piece to the puzzle by facilitating phylogenomic comparisons of cytoskeletal components with more well-studied organisms, and proteomic approaches to understanding *Giardia* cytoskeletal-mediated processes.

Researchers in the *Giardia* cytoskeleton field can draw on the lessons from *Chlamydomonas* (reviewed in Silflow and Lefebvre, 2001), *Toxoplasma* (reviewed in Morrissette and Sibley, 2002a,b) and *Trypanosoma* (reviewed in Kohl and

Gull, 1998), three protists in which a molecular understanding of the cytoskeleton is far more advanced. In particular, recent papers from the three systems point to the power of the approaches promoted in this review. Using GFP tagging and antisense knock-downs, Lechtreck et al. (2002) added significantly to prior knowledge concerning the function of SF-assemblin (the protein similar to β-giardin) in *Chlamydomonas* derived largely from biochemical studies. Synthesis of a variety of GFP constructs permitted a delineation of the C-terminal protein domain essential for assembly into striated microtubule-associated fibre; the integral role of SF-assemblin in the fibre formation was shown in studies in which cells with reduced SF-assemblin levels showed impaired flagellar assembly. Striated microtubule-associated fibre dynamics during mitosis were further studied by following full-length SF-assemblin-GFP protein throughout the cell cycle.

In the *Toxoplasma* cytoskeleton field, several recent papers explore unusual features of the tubulin cytoskeleton in this parasite. Tubulin was identified as a component of conoid fibres and could be seen assembling into novel non-symmetrical comma-shaped protofilaments during early stages of cell division using antibodies α-tubulin and yellow fluorescent protein-α-tubulin transfected-cells (Hu et al., 2002). An separate investigation of the role of two microtubule-organising centres in *Toxoplasma* using colchicine and oryzalin to disrupt microtubules, revealed that the parasite controls chromosomal segregation independently from cell shape and cytokinesis (Morrissette and Sibley, 2002a,b).

In *Trypanosome brucei* recent work by Hill et al. (1999, 2000) have identified a novel cytoskeleton protein (TLTF) that they postulate to define a larger class of cytoskeletal regulatory proteins. Synthesis of GFP-TLTF showed an association with the cytoskeleton throughout the cell cycle and localisation to electron dense regions in the flagellar pocket by immunoelectron microscopy via a novel targeting sequence. Similarity to a human protein previously identified as a candidate tumor-suppressor protein provides testable hypotheses in trypanosomes concerning possible regulatory roles for TLTF.

A recent proposal by Campanati et al. (2002) based on their morphologic observations provides a case in point for future research aims in *Giardia*. They observed short fibres extending from the funis and proposed that they may be dynein arms that act to move the funis and flagella. Testing of this provocative hypothesis will require identification of dynein in *Giardia*, localisation of dynein to the funis, and a targeted reduction of dynein levels to observe phenotypic effects and permit a corollary to be drawn between structure and function. Dynein regulatory signals, defined in other systems, can then be explored in *Giardia* to learn what cellular signals act to regulate the independent movement of the caudal flagella. Such paradigms can be similarly constructed for virtually every aspect of the *Giardia* cytoskeleton with respect to structure and function. Considerable work remains to be done.

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