

# What *Entamoeba histolytica* and *Giardia lamblia* tell us about the evolution of eukaryotic diversity

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*Entamoeba histolytica* and *Giardia lamblia* are microaerophilic protists, which have long been considered models of ancient pre-mitochondriate eukaryotes. As transitional eukaryotes, amoebae and giardia appeared to lack organelles of higher eukaryotes and to depend upon energy metabolism appropriate for anaerobic conditions, early in the history of the planet. However, our studies have shown that amoebae and giardia contain spliceosomal introns, ras-family signal-transduction proteins, ATP-binding cassettes (ABC)-family drug transporters, Golgi, and a mitochondrion-derived organelle (amoebae only). These results suggest that most of the organelles of higher eukaryotes were present in the common ancestor of all eukaryotes, and so dispute the notion of transitional eukaryotic forms. In addition, phylogenetic studies suggest many of the genes encoding the fermentation enzymes of amoebae and giardia derive from prokaryotes by lateral gene transfer (LGT). While LGT has recently been shown to be an important determinant of prokaryotic evolution, this is the first time that LGT has been shown to be an important determinant of eukaryotic evolution. Further, amoebae contain cyst wall-associated lectins, which resemble, but are distinct from lectins in the walls of insects (convergent evolution). *Giardia* have a novel microtubule-associated structure which tethers together pairs of nuclei during cell division. It appears then that amoebae and giardia tell us less about the origins of eukaryotes and more about the origins of eukaryotic diversity.

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## 1. Introduction

*Entamoeba histolytica* and *Giardia lamblia* are microaerophilic protists, which cause dysentery and diarrhea, respectively (figure 1) (Adam 2001; Huston and Petri 2001). Each is a single cell protist with a motile trophozoite stage and an immotile cyst stage. In many other ways amoebae and giardia are quite dissimilar. Amoebae have a single diploid nucleus, while giardia have two similar nuclei. While amoebae move along surfaces by an actin-myosin-mediated crawl, giardia swim by the synchronous beating of flagella and adhere to surfaces by means of a unique ventral disc (Ghosh *et al* 2001). The

cyst wall of amoebae is made of chitin (a polymer of N-acetyl-glucosamine), while the cyst wall of giardia is made of an alternative galactosamine-containing sugar polymer (Frisardi *et al* 2000). Although giardia is at the base of phylogenetic trees constructed of RNA or protein sequences, amoebae branch in an intermediate position that is not particularly early (Sogin and Silberman 1998; Baptiste *et al* 2002).

Amoebae and giardia resemble each other and *Trichomonas vaginalis*, a third microaerophilic protist, by the absence of enzymes of oxidative phosphorylation and the presence of fermentation enzymes, which closely resemble those of anaerobic prokaryotes (Müller 1993; Reeves

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Abbreviations used: ABC, ATP-binding cassettes; LGT, lateral gene transfer; MDR, multi-drug resistant.

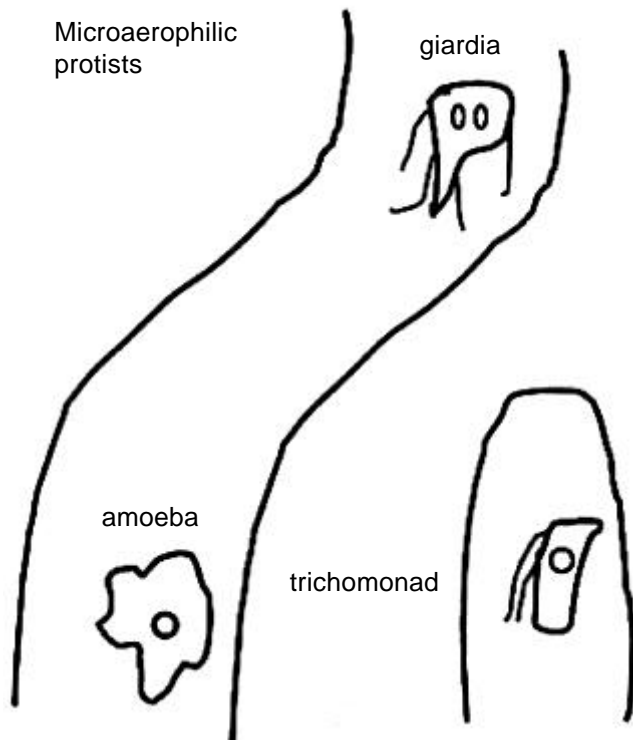
1984). These fermentation enzymes, which allow the protists to live under anaerobic conditions in the lumen of the bowel or vagina or in tissue abscesses, include numerous iron-sulfur proteins that are involved in converting pyruvate to acetyl-CoA and CO<sub>2</sub> (pyruvate : ferredoxin oxidoreductase or PFOR) or producing hydrogen gas (hydrogenase) (Rosenthal *et al* 1997).

The origins of the genes encoding these fermentation enzymes have been hotly debated. The biochemists, who identified many of these enzymes, speculated that they represented ancient eukaryotic energy metabolism prior to the acquisition of the mitochondrial endosymbiont (archaezoa or fossil hypothesis) (Reeves 1984; Müller 1993). In support of this idea, giardia and trichomonas each appear to branch early from the eukaryotic tree and numerous organelles present in higher eukaryotes appeared to be absent from these protists (mitochondria, Golgi, spliceosomal introns, and sexual stages) (Friend 1966; Adam 2001). Alternatively, it was suggested that these genes derive from an endosymbiont, which was an anaerobic bacterium such as a Clostridium (strict anaerobe hypothesis) or a facultative anaerobe such as an enterobacterium (facultative anaerobe or hydrogen hypothesis) (Martin and Müller 1998). The hydrogen hypothesis, suggested by the likely common ancestry of mitochondria and hydrogenosomes, is attractive because it gives a bio-

chemical explanation for the mitochondrial endosymbiosis (Müller 1993).

Finally, it has been suggested that the protists acquired genes encoding fermentation enzymes from prokaryotes by means of lateral gene transfer (LGT) (Rosenthal *et al* 1997; Field *et al* 2000; Nixon *et al* 2002a). One of the most important conclusions of whole genome sequences of > 50 prokaryotes of diverse types is that sets of genes encoding particular biochemical pathways have been frequently exchanged by means of LGT from one bacterium to another (Ochman *et al* 2000). An important corollary of this observation is that enzymes are conserved across bacterial species rather than being reinvented by each bacterium as the need arises.

This review will attempt to do two things. First, it will show how amoebae and giardia contain proteins and structures, which are similar to those of higher eukaryotes [e.g. spliceosomal introns, ras-family signal-transduction proteins, ATP-binding cassettes (ABC)-family drug transporters, Golgi, and a mitochondrion-derived organelle (amoebae only)]. These studies argue for a common eukaryotic ancestor, which was relatively complete from the point of view of cell biology. Second, we will review the evidence for LGT as an important mechanism for acquisition of protist genes encoding fermentation enzymes and a novel mechanism for generating eukaryotic diversity. In addition, we will show that the resemblance of the amoebic cyst wall lectins to plant and insect lectins most likely represents an example of convergent evolution. Finally, we will describe a novel microtubule-associated structure which giardia use to sort properly pairs of nuclei during cell division.



**Figure 1.** Luminal protists that parasitize man.

## 2. Introns of amoebae and giardia support the introns-late hypothesis

The first introns of amoebae and giardia were discovered by serendipity, when genes encoding proteins well-conserved across evolution [the amoebic cyclin-dependent kinase and the giardia 12 kDa (2Fe-2S) ferredoxin] lacked an in-frame start codon (Lohia and Samuelson 1993; Nixon *et al* 2002b). Although the absence of an in-frame start codon might have meant these protists genes were pseudogenes, RT-PCR in each case revealed a spliced product that encoded an intact protein. In the case of amoebae, this intron was the first of many that have subsequently been described (Willhoeft *et al* 2001). In contrast, this is the only giardia intron described to date, and it appears that giardia have many more genes encoding spliceosomal peptides than they have genes with introns (Sharp 1994). Phylogenetic analyses of spliceosomal peptides, which have been extensively duplicated and then differentiated, suggested that spliceosomes were

well-formed at the time that giardia branched from the eukaryotic tree (figure 2) (Nixon *et al* 2002b). The presence of introns in amoebae and giardia supports the intron-late hypothesis, which suggests introns are eukaryote-specific (Palmer and Logsdan 1991).

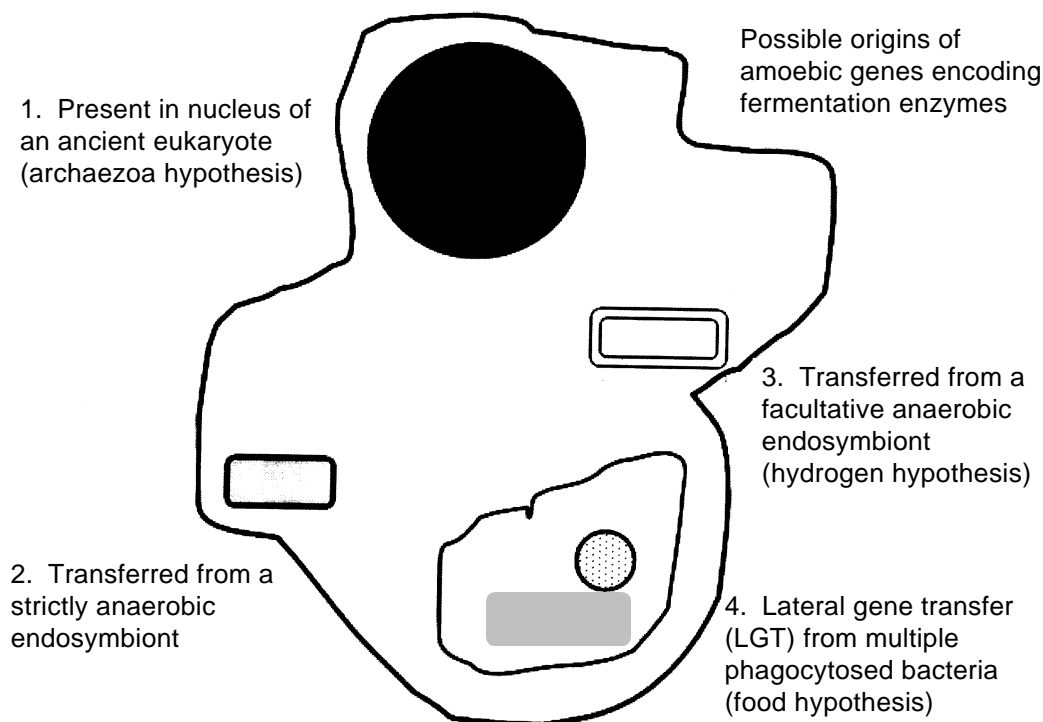
### 3. Signal-transduction proteins of amoebae and coincident evolution of phagocytosis

The presence of a cyclin-dependent kinase suggested amoebae may use signal-transduction proteins similar to those of higher eukaryotes (Lohia and Samuelson 1993). In a long-term collaboration with Anuradha Lohia of the Bose Institute in Kolkata, India, we identified numerous other amoebic genes encoding serine-threonine-dependent kinases and ras-family proteins, which are signal-transduction proteins in higher eukaryotes (figure 3) (Lohia and Samuelson 1994, 1996; Shen *et al* 1994). In addition, Sudip Ghosh, who is presently at the Indian Institute of Technology at Kharagpur, showed that an amoebic dominant mutant of  $p21^{racA}$  was defective in phagocytosis and in cell division (Ghosh and Samuelson 1997). Amoebic phagocytosis, like that of higher eukaryotes, was inhibited by wortmannin, which is an inhibitor of PI3-kinases. Tissue invasion is not under selection as no infective cysts are produced. In contrast, phagocytosis

of bacteria is selected, because it provides food for amoebae. It is likely, then, that tissue invasion is an example of coincident evolution (figure 4).

### 4. Amoebic ABC-family proteins, which resemble those of multi-drug resistant tumour cells, mediate emetine-resistance

The plant alkyloid emetine is used along with metronidazole to treat amoebic liver abscess (Samuelson 1999). With Esther Orozco of the National Polytechnic in Mexico City, we showed that emetine-resistant mutants of amoebae overexpress multiple genes encoding ABC-family proteins (figure 3) (Descoteaux *et al* 1995). ABC-family proteins, which are also known as P-glycoproteins or multi-drug resistant (MDR) proteins, contain twelve-transmembrane domains and two ABC. Gene amplification and overexpression of MDR proteins makes tumour cells resistant to numerous hydrophobic drugs (Gros *et al* 1986). Although the amoebic P-glycoprotein genes were not amplified, overexpression of mRNAs for three P-glycoproteins was associated with emetine-resistance. Further, Sudip Ghosh showed that overexpression of a single P-glycoprotein gene in transfected amoebae conferred emetine-resistance (Ghosh *et al* 1996).



**Figure 2.** Ideas concerning the origin of amoebic and giardia fermentation enzymes.

### 5. Amoebae have a cryptic mitochondrion-derived organelle (crypton) and Golgi

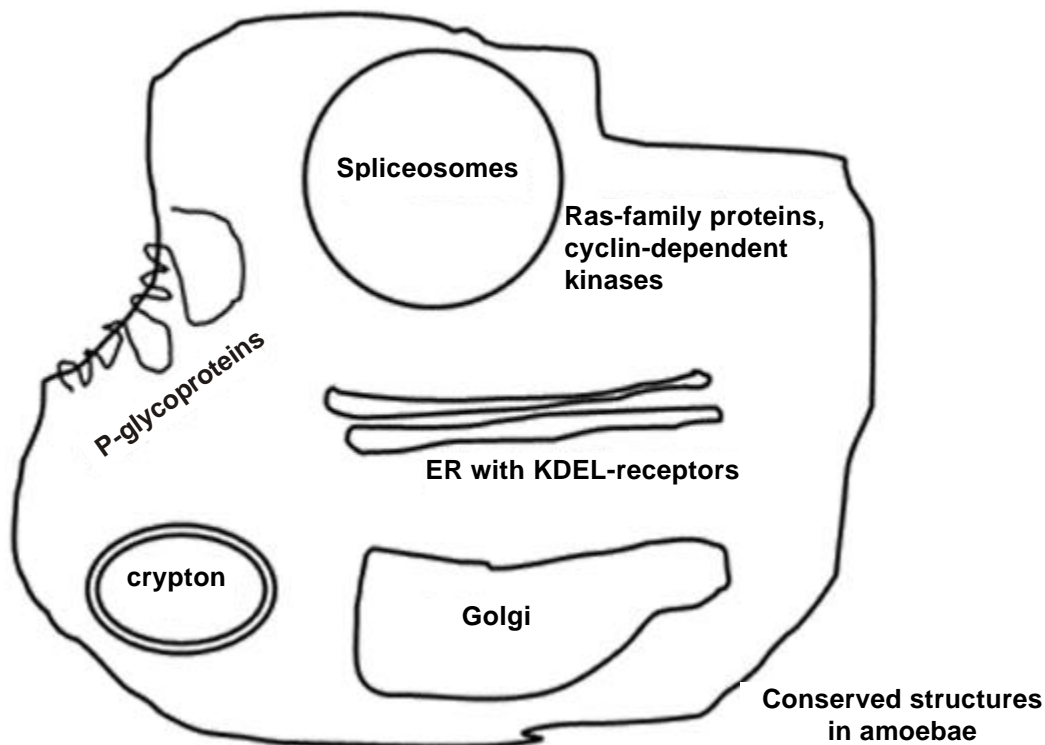
Amoebae and giardia lack enzymes of oxidative-phosphorylation, which are present in mitochondria of the vast majority of eukaryotes (Reeves 1984). Using methods similar to those that showed the hydrogenosome of *T. vaginalis* is actually a modified mitochondrion (Bui *et al* 1996), we identified a single atrophic mitochondrion-derived organelle in amoebae (figure 3) (Mai *et al* 1999). This organelle, which is bound by a double-membrane and may contain DNA, was named crypton, because it was hidden and its function remains unclear (Ghosh *et al* 2000). It is unlikely that the core energy metabolism is present within the crypton as is the case for mitochondria and hydrogenosomes, as the amoebic fermentation enzymes are all present in the cytosol (Reeves 1984; Müller 1993). Although giardia contains a few genes, which appear to be derived from the mitochondrial endosymbiont (e.g. Hsp60 12 kDa ferredoxin, and malic enzyme), this protist appears to lack an organelle (Roger *et al* 1998; Nixon *et al* 2002a,b). Similar methods were used to identify secretory vesicles, ER, and Golgi in amoebae (figure 3) (Ghosh *et al* 1999).

### 6. Most amoebic and giardia genes encoding fermentation enzymes derived from prokaryotes by LGT

The fermentation enzymes of amoebae, giardia, and trichomonas, which closely resemble those of anaerobic bacteria, are the target of metronidazole, the best drug against these protists (Samuelson 1999). Genes encoding the entire fermentation pathway of amoebae and giardia have been cloned, and phylogenetic methods have been used to show that the majority of these genes appear to have been acquired by the protists from anaerobic bacteria by means of LGT (figure 4) (Rosenthal *et al* 1997; Field *et al* 2000; Nixon *et al* 2002a). These prokaryotic genes appear to have been acquired by protists a long time ago because their sequences are relatively divergent. These results suggest the protists use LGT to adapt to their anaerobic environment. In contrast, LGT is extremely rare among higher eukaryotes, accounting for as few as 0.1% of human genes (Salzberg *et al* 2001).

### 7. Amoebic cyst wall chitin-binding lectins likely are an example of convergent evolution

The amoebic cyst wall, which protects parasites from



**Figure 3.** Structures common to higher eukaryotes in amoebae.

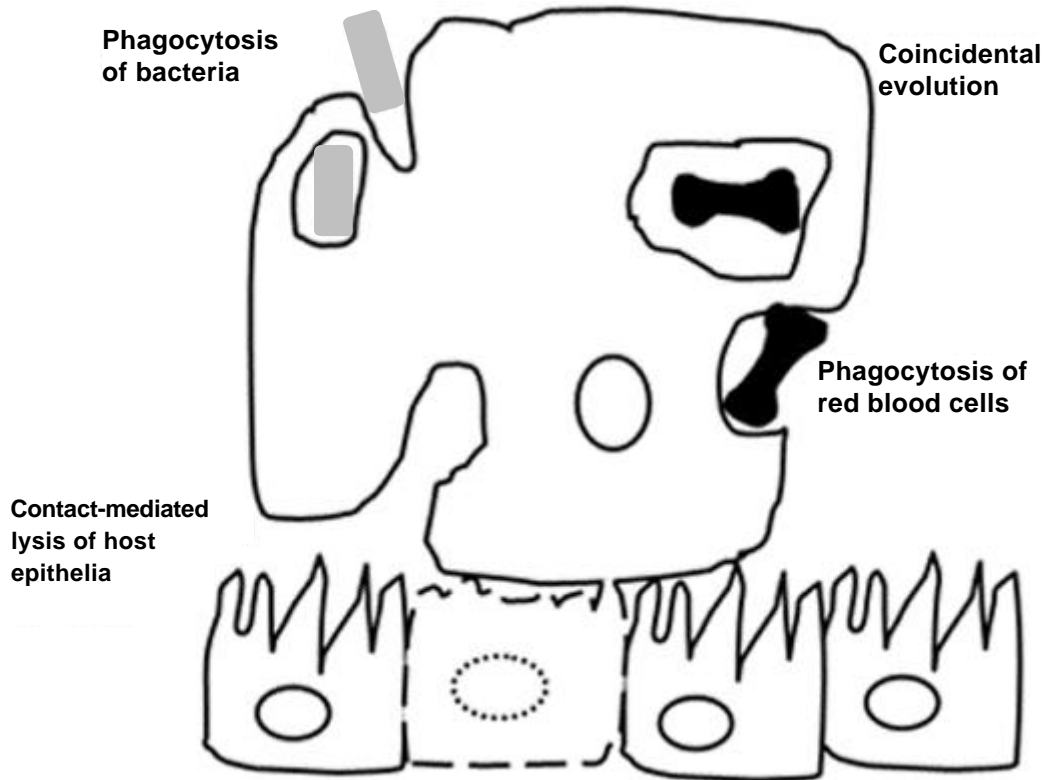


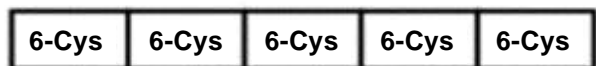
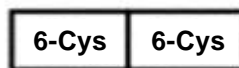
Figure 4. Coincident evolution of phagocytosis.

stomach acids, is composed of chitin fibrils and a single very abundant lectin called Jacob that cross-links the fibrils. Depending upon the *Entamoeba* species, the Jacob lectin is composed of two to five chitin-binding domains, each of which contains six conserved Cys residues (figure 5) (Frisardi *et al* 2000; Van Dellen *et al* 2002). Although insect peritrophins, which cross-link chitin fibrils in the wall formed around the blood meal, also have two to five chitin-binding domains containing six Cys residues; there is no other sequence similarity with the amoebic Jacob lectin (Shen and Jacobs-Lorena 1999). This is therefore an example of convergent evolution, which is extremely rare among proteins (Doolittle 1994). Similarly, amoebic chitinases have an N-terminal lectin domain, which contains an 8-Cys chitin-binding domain (de la Vega *et al* 1997; Van Dellen *et al* 2002) (figure 5). Although the plant lectins also contain 8-Cys chitin-binding domains, there is no sequence similarity with that of the amoebic chitinase (another example of convergent evolution).

**8. A novel microtubule-associated structure tethers together pairs of giardia nuclei**

*Giardia* adhere to host epithelia by means of a disc composed of microtubules and unique proteins called giardins

**Examples of convergent evolution**



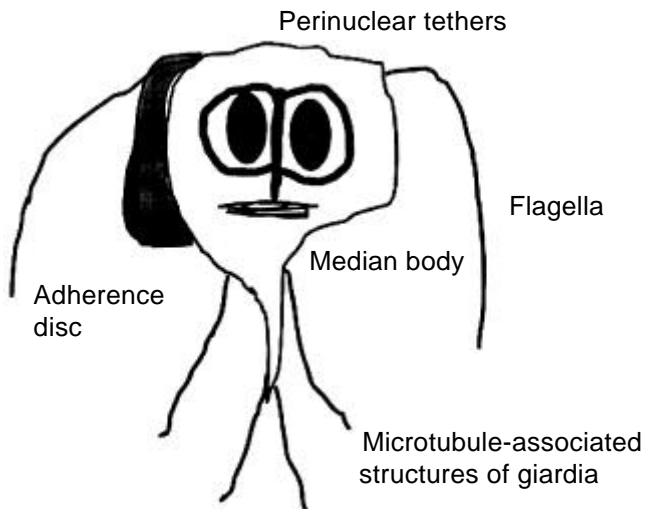
**Structures of Jacob lectins and peritrophins**



**Structures of amoebic and plant chitinases**

Figure 5. Convergent evolution of amoebic chitin-binding lectins.

(figure 6) (Holberton *et al* 1988). The two sides of *giardia* are held together by the median body, which is also composed of microtubules and an abundant unique protein called the median body protein (figure 6) (Marshall and Holberton 1993). We recently discovered a third



**Figure 6.** Microtubule-based structures of giardia.

microtubule-associated structure which tethers pairs of nuclei together during cell division (figure 6) (Ghosh *et al* 2001). These perinuclear microtubules, which are important for correctly distributing pairs of nuclei to mother and daughter giardia, are yet another example of eukaryotic diversity.

## 9. Conclusions

The presence within amoebae and giardia of structures common to higher eukaryotes (e.g. introns, signal-transduction proteins, P-glycoproteins, Golgi, and a mitochondrion-derived organelle) argues against the idea that these protists represent ancient, transitional forms. Instead the last common eukaryotic ancestor appears to have been relatively complete from the point of view of a cell biologist, although some functions (e.g. meiosis and alternative splicing) may have been absent. In contrast, the presence of relatively unique proteins within amoebae and giardia (fermentation enzymes, chitin-binding lectin, or perinuclear tethers) demonstrate the diversity of eukaryotes and suggest multiple mechanisms of eukaryotic diversification (e.g. LGT and convergent evolution).

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