The ‘primitive’ microaerophile Giardia intestinalis (syn. lamblia, duodenalis) has specialized membranes with electron transport and membrane-potential-generating functions

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INTRODUCTION

Some amitochondriate microaerophilic (Paget et al., 1989; Paget & Lloyd, 1990; Biagini et al., 1997a) flagellated protozoa have, until recently, been considered to be ‘ancient eukaryotes’ with ‘primitive’ characteristics (i.e. they lack mitochondria, peroxisomes, nucleoli, have only rudimentary Golgi stacks and produce energy by characteristically anaerobic pathways; Cavalier-Smith, 1987). Thus, the diplomonads, including Giardia intestinalis (syn. lamblia, duodenalis) and Hexamita inflata, and the parabasalia, e.g. Trichomonas vaginalis, are shown in textbook phylogenies (Madigan et al., 2000) as ‘early branching’ eukaryotes. However, this view has been challenged (Embley & Hirt, 1998), as all three species (G. intestinalis, H. inflata and T. vaginalis) have been shown to have genes encoding mitochondrial proteins. Thus, it is suggested that their apparently deep-branching status is a consequence of more recent secondary evolutionary modifications of aerobic mitochondriate organisms. Hydrogenosomes, the redox-active organelles of the parabasalia (Müller, 1998; Humphreys et al., 1998), of anaerobic rumen ciliates (Embley et al., 1995) and chytrid fungi (Biagini et al., 1997d), and of some free-living ciliates living in anoxic sediments (Biagini et al., 1997b), are now also believed to have been derived from ancestral mitochondria (Biagini et al., 1997c; Embley et al., 1997).

Alternative scenarios for the possible interactions of archaebal and bacterial precursors giving syntrophic and then symbiotic associations have been proposed (Gupta & Golding, 1996; Martin & Müller, 1998; López-Garcia & Moreira, 1999). Here we show, for the first time, that G. intestinalis has a small number of specialized plasma-membrane-associated structures that selectively partition the cationic, membrane-potential-sensitive fluorophore rhodamine 123. This organism also reduces a tetrazolium fluorogen at discrete plasma-membrane-associated sites. That these functions occur in distinctive specialized membrane systems supports the growing evidence that G. intestinalis may not be primitive, but is derived from an aerobic, mitochondria-containing flagellate.

METHODS

Materials. Fetal calf serum was supplied by GibcoBRL, through Life Technologies. Tryptone was purchased from Becton–Dickinson and trypsin was purchased from Bio-Mérieux. Rhodamine 123 and tetrabromorhodamine 123 were from Molecular Probes, through Cambridge Bioscience. 5-Cyano-2,3-ditolyl tetrazolium chloride (CTC) is a product of Polysciences, distributed through Park Scientific. Fixatives and resins were obtained from Agar Scientific. All other materials were supplied by Sigma.
Growth of organisms. *G. intestinalis* strain Portland-1 (ATCC 30888), originally described by Meyer (1976), was a gift from Michael R. Edwards, University of New South Wales, Sydney. Trophozoites were cultured axenically and anaerobically in screw-capped Nunclon tubes (Life Technologies) at 37 °C on Diamond’s modified TYI-S-33 medium containing 2% tryptone, 1% yeast extract, 0.5% glucose, 0.106% arginine, 0.2% NaCl, 0.1% K$_2$HPO$_4$, 0.06% KH$_2$PO$_4$, 0.1% cysteine, 0.1% bovine bile, 0.02% ascorbic acid, 0.0023% ammonium ferric citrate and 3% (v/v) minimal essential medium with Earle’s salt (Keister, 1983; Edwards et al., 1989) supplemented with 10% (v/v) heat-inactivated fetal calf serum. Subculturing was performed routinely at 48 h intervals, by replacing the spent medium for fresh without detaching the cell monolayer. Cells were harvested by replacing the spent medium with fresh, chilling the tubes on ice for 20 min and then inverting them gently to detach the monolayer. Cells were counted on a Fuchs–Rosenthal haemocytometer slide with 0.4% (w/v) Trypan blue as the viability indicator; typically this gave cell numbers of 2 × 10⁶ ml⁻¹.
Fluorescence microscopy. Cells were harvested and washed twice in PBS (0.08 M KH$_2$PO$_4$; 5 mM K$_2$HPO$_4$; 150 mM NaCl; pH 7.4) to remove any residual medium, then resuspended in 0.5 ml PBS containing 5.5 mM glucose. The fluorophore, rhodamine 123, was added (10 µl of 1 mg ml$^{-1}$) to the cell suspensions and they were incubated for 15 min at 22°C. The suspensions were then washed twice in PBS and resuspended in 200 µl PBS. Cells were mounted and viewed using an Olympus BH2 triocular fluorescent microscope. Images were captured on Fuji ISO 400 (daylight) 38 mm film.

Confocal laser scanning microscopy. Cells were incubated with rhodamine 123 (as for the fluorescence microscopy) but in 0.31 M mannitol instead of PBS, or they were incubated with CTC for 1 h before analysis to allow location of electron transport components. The cells were then viewed using a Molecular Dynamics Sarastro 2000 confocal laser scanning microscope. Specimens were scanned by using a 25 mW argon laser, with the appropriate excitation and emission filters. Specimens were examined using oil emersion objectives, ×60 (50 µm confocal aperture) and ×100 (100 µm confocal aperture). Series sections through samples were taken at 1 µm intervals (512 × 512 pixels; ∼0.5 µm thick), and three-dimensional constructs were prepared using Molecular Dynamics Volume Workbench, running in a Silicon Graphics UNIX workstation.

X-ray microanalysis. Cells in growth medium were fixed in 1.25% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 5 min at 4°C. After washing twice in the same buffer, the cells were resuspended in 50 µl of tetrabromorhodamine 123 (1 mg ml$^{-1}$ in methanol), incubated at room temperature for 30 min and then washed twice. After fixation for a further 5 min at 4°C followed by three washes in water, cells were washed for 20 min in 70% ethanol at 4°C. Freeze-substitution (AFS, Reichert) was programmed to give a temperature increase from −25°C to room temperature over 52 h. Dehydration of the cells in 100% ethanol (90 min) was followed by embedding them in a graded series of Lowicryl HM20 resins (50, 70 and 95% in pure ethanol, respectively, for 30 min each), then washing them twice with 100% Lowicryl for 30 min. Pure Lowicryl was polymerized under UV light for 48 h, during which time the temperature was increased gradually from −25°C to room temperature. Sections, 0.1 µm thick, were cut using glass knives on a Reichert Ultracut microtome; these were then mounted on pioloform-coated copper grids. The sections were analysed using a JEOL 1210 transmission electron microscope equipped with an energy-dispersive X-ray analyser (ISIS system). X-ray dot mappings of Br were collected over 2 h, and the spectra were obtained over 100 s. The characteristic X-ray emission peaks of Br Kα were detected at 1192 keV.

RESULTS

Fig. 1(a) shows the confocal laser scan of a single section of Giardia intestinalis after exposure to the cationic, membrane-potential-sensitive dye rhodamine 123. In this figure, it can be seen that the fluorophore penetrated the plasma membrane and was selectively taken up into plasma-membrane-associated structures, which appear, for the most part, as spherical areas (1.34 ± 0.1 µm, n = 12), but in some cases appear as oval areas (1.96 ± 0.2 µm × 1.1 ± 0.1 µm, n = 5). The single sections (thickness 0.46 µm) showed, on average, about five brightly fluorescent ‘patches’ per organism; the fluorophore was also more generally localized around the inner limit of the plasma membranes, especially at the anterior end of the organism, where it shows up as a bright rim. A series of sections (imaged on an artificial colour scale, Fig. 1b) and a three-dimensional reconstruction (not shown) indicated that dye accumulation was predominantly confined to internal organelles, but it also accumulated in the perinuclear region. Confocal laser scanning of a single section (Fig. 2) indicated, on an artificial colour scale, the intensity and distribution of fluorescence. An
independent method for imaging the foci of cationic-dye uptake, using energy-dispersive X-ray analysis of the Br Kα emission peaks of 2′, 4′, 5′, 7′-tetrabromorhodamine 123 bromide incorporated into the organism, was employed. The X-ray energy spectrum for this method is shown in Fig. 3(a). Again, the X-ray dot mappings (Fig. 3b) show patchy deposition of the dye in distinct areas of the section, and it is specifically located in areas immediately inside the plasma membrane.

Incubation of intact organisms with 4.4 mM glucose and CTC for 1 h gave discrete locations where the insoluble, red-fluorescent formazan reduction product was deposited (Fig. 1c). The rate of dye reduction was enhanced in freeze–thawed organisms by the inclusion of 0.1 mM NAD(P)H in the incubation medium. Confocal scanning laser microscopy showed that the electron transport activity was specifically located to discrete areas (Fig. 1d) that line the inner face of the plasma membrane, which appear in section as a rim around the inside of the organism. A series of sections is also shown for this confocal scanning laser microscopy in Fig. 2. Rotation of a three-dimensional reconstruction (not shown) further confirmed this localization.

**DISCUSSION**

*G. intestinalis* was once grouped with other amitochondriate protozoa in a kingdom, the Archezoa (comprising the Metamonada, Microsporidia, Parabasalia and the Archamoebae), as it was assumed that these anaerobic organisms were closely related to the early predecessors of all higher organisms. The hypothesis that led to the proposal that this kingdom represents the most primitive and ancient extant eukaryotes (Keeling, 1998) found support from work on RNA (Boothroyd et al., 1987; Edlind & Chakraborty, 1987; Sogin et al., 1989), which suggested that *G. intestinalis* should be located on the deepest branch of the eukaryotic phylogenetic tree; several sequences, including those of elongation factors 1 and 2, seemed to confirm this view (Hashimoto et al., 1995). Now, evidence has been accumulated which suggests that many of the archaean lineages evolved after the mitochondrial symbiosis (Keeling, 1998), and this indicates that these amitochondriate organisms once had mitochondria derived from an endosymbiont-containing progenitor (Solys & Gupta, 1994; Horner et al., 1996; Germot et al., 1996; Roger et al., 1996). Thus, a mitochondrial-like chaperonin 60 gene (Roger et al., 1998) and a gene for mitochondrial valyl-tRNA synthetase (Hashimoto et al., 1998) in the nucleus of *G. intestinalis* are clearly related to mitochondrial homologues from other eukaryotes. Furthermore, it is suggested that these genes are relics of an ancient organelle. The phylogenetic evidence for the emergence of diplomonads within a clade of mitochondria-bearing organisms has been reviewed critically and in detail by Philippe et al. (2000).

Hydrogenosomes are also now believed to have been derived from a mitochondria-like ancestral organelle (Biagini et al., 1997c; Embley et al., 1997) as well as evidence from their molecular biology, they share some structural characteristics (inner and outer membranes, Finlay & Fenchel, 1989) and functions [sequestration of Ca²⁺ (Chapman et al., 1985; Humphreys et al., 1998; Biagini et al., 1997c, d) and transmembrane electrochemical potential (Humphreys et al., 1998) with mitochondria. The discovery of a ciliate hydrogenosome with DNA that shows high sequence similarity with mitochondrial SSU rRNA genes from aerobic ciliates (Akhmanova et al., 1999) confirms the mitochondrial origin of hydrogenosomes.

Mitochondrial remnants have been described in the anaerobic parasite *Entamoeba histolytica* (Rodrigueze et al., 1998). An organelle bound by two membranes has been discovered in this organism by using antibodies raised against α protein (cpn 60), which is specific to mitochondria (Tovar et al., 1999; Mai et al., 1999). These drastically modified remnants of the redox organelle, like hydrogenosomes, are an evolutionary
consequence of attenuated function in low-\(O_2\) environments.

Rhodamine 123 is a cationic, membrane-potential-sensitive dye, which has been used extensively to locate mitochondria. It is electrophoresed through plasma membranes and then from the cytosol into the mitochondrial matrix space – in energized mitochondria, the ratios of fluorophore concentrations may reach \(10^4\): \(10^2\): 1 between the intra- and extra-mitochondrial spaces and the extra-cellular suspending fluid, respectively (Chen, 1988). Rhodamine 123 has been used to stain mitochondria specifically in aerobically grown yeast (Lloyd et al., 1996), and it also accumulates in the hydrogenosomes of *Trichomonas vaginalis* (Harris, 2001). Similar specificity of uptake of the cationic cyanine dye dihexyloxycarbocyanine has been demonstrated in these hydrogenosomes (Humphreys et al., 1998), and its uptake has also been demonstrated in the free-living, ‘anaerobic’ ciliate *Metopus contortus* (Biagini et al., 1997c). Our observations of structures with mitochondria-like functions in *G. intestinalis* further suggest the loss of fully fledged mitochondria from this parasite and the secondary nature of its mitochondrial status. The particulate nature of NAD(P)H- and pyruvate-driven electron transport chains has previously been demonstrated by subcellular fractionation after gentle mechanical disruption (Ellis et al., 1993). Subcellular fractionation, after gentle mechanical disruption, of *G. intestinalis* has indicated that pyruvate: ferredoxin oxidoreductase, and NADH and NADPH oxidoreductases are mostly non-sedimentable (73%, 57% and 68%, respectively) after centrifugation at 100000 \(g\) for 1 h (Ellis et al., 1993). The acceptors used in that study were methyl viologen for pyruvate-driven electron transport and FMN for the nicotinamide-nucleotide-linked activities. The fluorogenic tetrazolium used in the present study acts as an electron acceptor for all three donors. In organisms with mitochondria, this compound, like other tetrazolium salts with redox potential values similar to CTC (Lloyd et al., 1993), has been used as a cytological marker for respiratory chain activities of the inner-mitochondrial membrane.

Extensive studies using transmission electron microscopy have not yet positively identified the nature of the structures revealed by optical methods. Multi-lamellar residues were present in some of the organisms, and their frequency of occurrence was greater after controlled exposure to low concentrations of \(O_2\) (Lloyd et al., 2000; Harris, 2001). Further work is necessary to establish the precise physiological functions of the discrete membrane structures, shown by the fluorescence methods used here, and those of multi-lamellar bodies.

Electron transport and the development of membrane potential are characteristic activities of the inner-mitochondrial membrane. We might suggest that although the habitat of the trophozoite is a low-\(O_2\) environment and its energy metabolism uses characteristically anaerobic routes, not all mitochondrial functions are dispensable. The consequent structure/function relationships of the specialized membrane elements that perform the redox-active and trans-membrane electrochemical-potential-generating activities described here may be considered as the solution that has been evolved in an organism where there are no longer fully fledged mitochondria (Lloyd et al., 1983).

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**REFERENCES**


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