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Cardiolipin, a lipid found in mitochondria, hydrogenosomes and bacteria was not detected in *Giardia lamblia*

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ABSTRACT

Giardia lamblia is a protozoan parasite with many characteristics common among eukaryotic cells, but lacking other features found in most eukaryotes. Cardiolipin is a phospholipid located exclusively in energy transducing membranes and it was identified in mitochondria, bacteria, hydrogenosomes and chloroplasts. In eukaryotes, cardiolipin is the only lipid that is synthesized in the mitochondria. Biochemical procedures (TLC, HPLC) and fluorescent tools (NAO) were applied in order to search for cardiolipin in *G. lamblia*. In addition, BLAST searches were used to find homologs of enzymes that participate in the cardiolipin synthesis. Cardiolipin synthase was searched in the *Giardia* genome, using *Saccharomyces cerevisiae* and *Mycoplasma penetrans* sequences as bait. However, a good match to *G. lamblia* related proteins was not found. Here we show that mitosomes of *G. lamblia* apparently do not contain cardiolipin, which raises the discussion for its endosymbiotic origin and for the previous proposal that *Giardia* mitosomes are modified mitochondria.

1. Introduction

In developing countries giardiasis is among the 10 most common infections affecting humans (Pawlowski, 1984; Huang and White, 2006) and is widely prevalent in children, causing diarrhea and malnutrition. *Giardia* presents the trophozoite form, which colonizes the small intestine and in the gut lumen trophozoites differentiate into cysts, which pass out with the host's feces. Studies carried out in recent years have shown that organelles such as mitochondria, peroxisomes, and Golgi complex are absent (or controversial) in trophozoites (Reiner et al., 1990; Lujan et al., 1995; Lanfredi-Rangel et al., 1999; Marti and Hehl, 2003).

The discovery of mitochondrion-related organelles such as hydrogenosomes (Lindmark and Muller, 1973) and mitosomes (Mai et al., 1999; Tovar et al., 1999, 2003) in eukaryotes that lack typical mitochondria has revived interest concerning the origin and evolutionary aspects of the endosymbiosis-derived organelles of eukaryotes.

Mitosomes are bounded by double-membranes and are found in unicellular eukaryotes, including *Entamoeba histolytica* (Mai et al., 1999; Tovar et al., 1999) and microsporidians (Williams et al., 2002). Mitosomes were identified in *Giardia lamblia* (Tovar et al., 2003) and thus, *Giardia* contains these remnant organelles. They are bounded by double-membranes which play a role in iron–sulfur protein maturation and other functions mainly related to mitochondria. Some groups claimed that this finding could indicate that *Giardia* is not primitively amitochondrial and that it has retained a functional organelle derived from the original mitochondrial endosymbiont ancestral (Tovar et al., 2003).

Cardiolipin (CLP[1]) is a phospholipid located exclusively in energy transducing membranes. It is a typical component of bacterial plasma membrane and is also present in the inner mitochondrial and in chloroplasts membranes (Maftah et al., 1989), and in hydrogenosomes (de Andrade Rosa et al., 2006). Since cardiolipin was demonstrated in these cell structures, its identification in mitosomes would certainly be expected. In eukaryotes this is a lipid that is synthesized in the mitochondrion, where it remains throughout the life of the cell. Therefore, the presence of CLP in mitosomes would be an additional argument for the hypothesis of a closer proximity with mitochondria and the symbiotic origin of this organelle.

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[1] *Abbreviations used:* CLP, cardiolipin; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatograpy; NAO, 10-*N*-nonyl acridine orange; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; PBS, phosphate-buffered saline; ADP, adenosine diphosphate; ATP, adenosine triphosphate; DNA, deoxyribonucleic acid; PLDc, phospholipase D; PG, phosphatidylglycerol; CDP-DAG, CDP-diacylglycerol; TMP, Trans Membrane Potential.

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In an attempt to point out one more biochemical similarity between mitochondria and *Giardia* mitosomes, this work aims to identify cardiolipin in such structures, applying the techniques that were used for the identification of CLP in *Tritrichomonas foetus* hydrogenosomes recently published (de Andrade Rosa et al., 2006).

2. Material and methods

Cell cultures. G. lamblia strain WB (American Type Culture Collection, No. 30957) was cultivated in TYI-S-33 medium enriched with 10% heat-inactivated fetal bovine serum at pH 7.05, without added vitamins, iron, or antibiotics, but supplemented with 0.1% bovine bile (Keister, 1983) for 48–72 h, at 37 °C. Cell cultures of *T. foetus* (de Andrade Rosa et al., 2006), a cattle parasite harboring hydrogenosomes and *Saccharomyces cerevisiae* (Pereira-Neves and Benchimol, 2007), which are mitochondria-harboring organisms, were also used as control in all the experiments. *G. lamblia* and *T. foetus* were used at a concentration of 6×10^6 and 4×10^6 cells/ml, respectively, for TLC experiments.

Fluorescent Detection of CLP using 10-N-nonyl acridine orange (NAO). (Gallet et al., 1995). Living cells were stained with different concentrations of NAO (Molecular Probes, Eugene, OR), 100 nM, 500 nM, 1 μM, and 5 μM, for 10–15 min and examined in an Axiophot II Zeiss microscope.

2.1. Mitochondria staining

5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (Bernardi et al., 1999) and Mitotracker (Molecular Probes, Eugene, OR) are selective dyes for polarized mitochondria membranes under appropriate loading conditions, where they fluoresce with an intensity proportional to the $\Delta\Psi$m. Living cells were washed twice in phosphate-buffered saline (PBS) and further immobilized on a cover glass treated with poly-L-lysine. Afterwards, the cells were stained with 2 μM JC-1 for 30 min at 37 °C or 1 μM Mitotracker for 15 min at 37 °C. The samples were observed under a Zeiss Axiophot II microscope (Zeiss) and images were acquired and processed using a chilled C5985-10 CCD camera (Hamamatsu, Japan).

2.2. Preparation of mitosomes fraction

One liter of cells were chilled and harvested by centrifugation in a Sorvall RC B ultracentrifuge (SS-34) at 1000*g* for 10 min and washed three times by centrifugation in ST buffer containing 250 mM sucrose, 0.5 mM KCl, 10 mM Tris–HCl, 0.5% Triton X-100, pH 7.2. The pellet was resuspended in 10 ml ST buffer with protease inhibitor cocktail (Sigma Co., USA) and disrupted in a potter-type homogenizer with teflon pestle. The resulting homogenate was submitted to differential centrifugation. The first centrifugation was at 680*g* for 10 min and next, 2760*g* for 20 min. The resulting pellet containing unbroken cells and cytoskeleton was discarded. The supernatant was centrifuged at 50,000*g* for 30 min in a Beckman ultracentrifuge using a type 65 rotor. The pellet, corresponding to an enriched mitosome fraction, was resuspended in 0.5 ml of ice-cold ST buffer.

2.3. Lipid extraction and separation by thin layer chromatography (TLC)

Total lipids were extracted from *G. lamblia* (whole cells) *T. foetus*, enriched mitosomes fraction and hydrogenosomes. In brief, the lipid extraction started by adding 4 ml of chloroform:methanol:HCl (2:1:0.075, v/v) to the samples in glass tubes (Horwitz and Perlman, 1987). After the complete procedure, the resultant organic phase was dried under N_2 gas, quantified gravimetrically and then reconstituted in 90 μl of chloroform: methanol:H_2O (7.5:2.5:0.2 v/v). Total lipids were separated by unidimensional TLC (Horwitz and Perlman, 1987). After the chromatographic procedure, the TLC plate was kept in an exhaustion chamber for approximately 1 h to allow solvent evaporation. Then the TLC plates were developed in an iodine vapor atmosphere and the lipids of interest were identified comparing the relative mobility of the spots with the commercial standards used. The spot corresponding to CLP, identified by the relative mobility in the solvent system used, was scraped from the TLC plate and CLP extracted from the silica after three washes with chloroform. The CLP-containing sample was dried under N_2 and used for HPLC and mass spectrometry analysis as follows. CLP (MW = 1472) from Avanti Polar Lipids (Philadelphia, PA, USA) was used as standard.

2.4. High performance liquid chromatography (HPLC)

HPLC analysis was performed according the method recently described (Singh et al., 2005) using a Shimadzu SCL-10AVP System connected to a UV detector (model LC-10AT, Shimadzu) with 100 μl loop. The column used was a LiChroCart® 4-4 LiChrosphere® 100 RP-18 5 μM HPLC (Merck). The CLP standard and samples analysis were obtained by elution with isocratic KH_2PO_4 buffer (pH 2.7, 50 mM)-methanol (85:15, v/v) and were detected at UV 205 nm. The flow rate was 1.0 ml/min.

2.5. Bioinformatics

In order to search for sequences with a best match to CLP synthase, we searched sequence-data in *G. lamblia* protein database, using the sequence of the CLP synthase already identified in *S. cerevisiae* and *Mycoplasma penetrans* as bait, using the NCBI-blastp and T-Coffee multiple alignment program.

3. Results

3.1. Fluorescent detection of CLP

The subcellular localization of NAO with different concentrations was determined by fluorescent microscopy (Fig. 1[2]). Green fluorescence was observed in the nuclear envelopes, on the endoplasmic reticulum and in vacuoles, some of which resembled mitosomes due to their localization. When mitochondrial stains were used, as Mitotracker and JC-1, the fluorescence had a similar distribution for that obtained with NAO (not shown). *Tritrichomonas foetus* (Fig. 2a), a parasite protozoon that presents hydrogenosomes and *S. cerevisiae* that contains mitochondria, were used as positive control, since both organelles present cardiolipin. Hydrogenosomes (Fig. 2b) and mitochondria of *S. cerevisiae* (Fig. 2c) were stained in red, indicative of functional organelles.

3.2. Thin layer chromatograph (TLC)

Thin layer chromatography was performed using lipid extracts obtained from whole cell homogenate of *G. lamblia*, *T. foetus*, enriched mitosomes fraction, hydrogenosomes and commercial cardiolipin as a standard (Fig. 3). Fig. 3 shows a representative TLC plate after exposure to iodine vapors, where one can observe some of the major phospholipids (phosphatidylserine, phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine) that are present in the samples tested. The CLP spot was not detected in *G. lamblia* after iodine vapor exposure, although this spot was seen

[2] For interpretation of color mentioned in this figure the reader is referred to the web version of the article.