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The mitochondrion in dividing *Leishmania tarentolae* cells is symmetric and circular and becomes a single asymmetric tubule in non-dividing cells due to division of the kinetoplast portion

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Abstract

Kinetoplastid protozoa have a single mitochondrion that extends throughout the cell. The disk-shaped portion of the mitochondrion adjacent to the basal body of the flagellum contains the kinetoplast DNA nucleoid body which consists of thousands of catenated minicircles and a smaller number of catenated maxicircles. The maxicircles contain structural genes and cryptogenes, rRNA genes, and a few guide RNA genes The minicircles contain the majority of the guide RNA genes. The long slender non-dividing stationary phase *Leishmania tarentolae* cells in culture have an asymmetric mitochondrion that consists of a single tubule extending from one edge of the kinetoplast portion. This presents a problem for cell division, in that one daughter cell will receive significantly less mitochondrial membranes than the other cell. We show in this paper that the solution to this problem is that dividing cells, which are normally shorter and rounder than stationary phase cells, possess a symmetric circular mitochondrion that has mitochondrial tubules extending from both edges of the kinetoplast which are joined in the posterior region of the cell. This implies that growth of the mitochondrion occurs after cell division, either from elongation of the longitudinal tubule towards the anterior of the cell, or from elongation of the kinetoplast portion of the mitochondrion towards the posterior region and fusion of the tubules. © 1997 Elsevier Science B.V.

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1. Introduction

Kinetoplastid protozoa appear in general to contain a single mitochondrion which extends throughout the cell [1–3]. The strongest evidence for this has come from three dimensional reconstructions in *Crithidia fasciculata*, *Blastocrithidia culicis* and *Trypanosoma cruzi*, based on serial thick sections and high-voltage transmission electron microscopy [4–6]. The portion of the mitochondrion adjacent to the basal body of the flagellum, which is termed the kinetoplast, contains the kinetoplast DNA (kDNA) nucleoid body [1]. The kDNA nucleoid body is composed of a highly structured single network of thousands of catenated minicircles, which encode guide RNAs (gRNAs), and a smaller number of catenated maxicircles [7–12], which encode mRNAs and also a small number of gRNAs. Replication of the kDNA, which occurs during the nuclear S phase [13–17], results in the doubling of the length of the kDNA nucleoid body as visualized by transmission electron microscopy in longitudinal sections or by light microscopy of DAPI-stained live cells or Giemsa-stained fixed cells. Division of the kinetoplast occurs prior to nuclear division in most cells by pinching together of the mitochondrial membranes and segregation of the kDNA nucleoid body into two portions. Cytokinesis then occurs, resulting in two daughter cells.

In stationary phase *L. tarentolae* promastigote cells in culture, the apparently paradoxical observation has been made that the single mitochondrion is asymmetric, extending from one side of the kinetoplast portion down past the nucleus to the posterior portion of the cell [18]. This asymmetric organellar configuration would present a severe problem at cell division since it would result in a loss of a substantial portion of the mitochondrial membrane material in one daughter cell. Both daughter cells would retain a full complement of the structural and rRNA-encoding maxicircle DNA molecules and the guide RNA-encoding minicircle DNA molecules, but one cell would lack the entire tubule of the single mitochondrion extending from the kinetoplast region and the associated enzymatic machinery. This would have to be regenerated in that cell prior to cell division.

We present in this paper a solution of this apparent paradox by showing that the mitochondrion in dividing cells is symmetric and appears to be circular, and that the observed asymmetric organization is produced by division of the mitochondrion at the kinetoplast region.

2. Materials and methods

2.1. Cell culture and light microscopy

L. tarentolae cells were grown with gentle agitation in brain heart infusion medium (Difco) at 27°C. The cells were centrifuged at 1000 × *g* for 10 min and the pellet resuspended in 0.25 M sucrose, 10 mM Tris–HCl (pH 7.5), 1% bovine serum albumin (Fraction V, Sigma), and a 1–5 μl aliquot rapidly air-dried on a glass slide, fixed for 5 min in cold methanol and stained for 30 min in Giemsa (Sigma) diluted 1:20 with 0.01 M Tris–HCl (pH 7.5). Slides were rinsed with water and air-dried. Micrographs were taken using a 100 × apochromatic objective.

2.2. Electron microscopy

The methods used for preparation of the cells for transmission electron microscopy were described previously [19]. Stationary phase non-dividing cells were embedded in agarose, and pieces of the agar-pellet were fixed in glutaraldehye and osmium tetroxide, post-stained with uranyl acetate, and embedded in Vestopal. Continuous ribbons, comprised of 20–30 serial sections each about 50 nM thick, were cut and collected intact on one-hole grids, as described previously [20]. Sections were stained with uranyl acetate and lead citrate.

2.3. Immunofluorescence and rhodamine 123 staining

The procedure has been described previously [18,21,22]. The cells were fixed with in 2% paraformaldehyde in 0.15 M NaCl/5 mM K phos-

phate (pH 7.4) (PBS) for 6 min at 25°C, extracted with 0.1% Triton X-100 for 10 min, and active aldehyde groups neutralized with 0.1 M glycine for 10 min. The cells were washed in PBS and let adhere to polylysine-treated slides (1 mg ml^{-1} poly(L-lysine) for 15 min). The slides were blocked with 20% goat serum/0.1% Tween-20 in PBS for 30 min, washed with PBS, and incubated with p18 antiserum (1:1000) or pre-immune serum and then with fluorescine isothiocyanate (FITC)-conjugated goat anti-rabbit second antibody (500 μg ml^{-1}, diluted 1:50) for 30 min. The slides were then washed with PBS-Tween and stained with 0.4 μg ml^{-1} DAPI. The slides were mounted with anti-fade solution (10% glycerol/0.1 M NaCl/50 mM Tris–HCl (pH 9.0)/1 mg ml^{-1} *p*-phenylenediamine (Fisher)/4 μl ml^{-1} 1 N NaOH), and cover slips sealed with clear nail polish. The cells were observed using in a Nikon UV microscope the appropriate excitation and barrier filters and micrographs taken with T-Max p3200 film.

To visualize the mitochondrion using uptake of rhodamine 123, 40 μg ml^{-1} dye was added to a cell culture, which was incubated at 27°C for 10 min. The cells were then pelleted and washed twice at 5°C in 0.15 KPO4 (pH 7.4), resuspended in 0.25 M sucrose-1% bovine serum albumin (Sigma), and spread onto a glass slide, rapidly air-dried and fixed in methanol for 5 min. It was necessary to air-dry the cells to prevent rapid loss of rhodamine fluorescence upon ultra violet (UV) illumination. The cells were observed in a Nikon UV microscope and micrographs taken with T-Max p3200 film.

3. Results

3.1. Cell division of L. tarentolae cells in culture

Representative micrographs of division forms of Giemsa-stained *L. tarentolae* cells in an asynchronous log phase culture are shown in Fig. 1, arranged in a proposed temporal sequence. The initial event is usually the division of the kinetoplast nucleoid body (1,2), which is followed by the division of the nucleus (3,4). The cells then undergo cytokinesis and pull apart in the anterior region.

In asynchronous cultures, cells in early log phase are generally more rounded than stationary phase cells and have short or no flagella. As the cells approach stationary phase, they become long and slender and have long flagella.

3.2. Three dimensional reconstruction of the single mitochondrion in stationary phase L. tarentolae cells in culture.

A three dimensional reconstruction of the contours of the outer membrane of the single mitochondrion in a stationary phase *L. tarentolae* cell in culture was performed from transmission electron micrographs of serial sections. Several sets of serial sections through different cells showed approximately the same profile and this was chosen as representative. Three views of the reconstruction are shown. The kinetoplast portion of the mitochondrion which contains the kDNA is indicated (k), as well as the location of the nucleus in the cell (n). Note the existence of a narrow mitochondrial tubule extending from one end of the kinetoplast region past the nucleus, and the expansion of the tubule into a disk-like structure extending into the posterior portion of the cell.

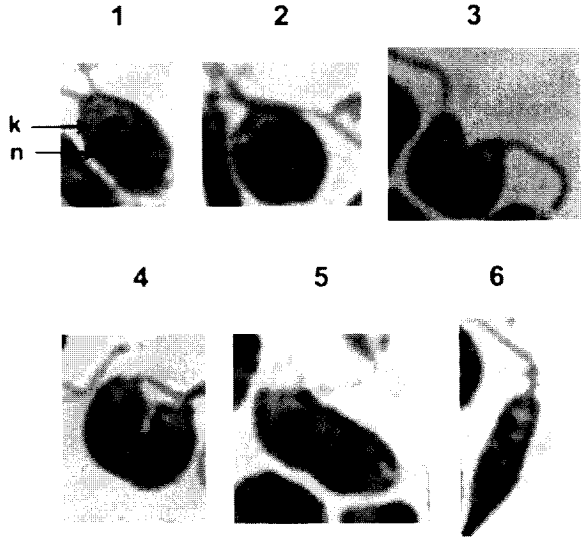

Fig. 1. Representative micrographs of Giemsa-stained *L. tarentolae* cells illustrating different stages of cell division. The images are arranged in a proposed temporal sequence (1–6). k, kDNA nucleoid body; n, nucleus.

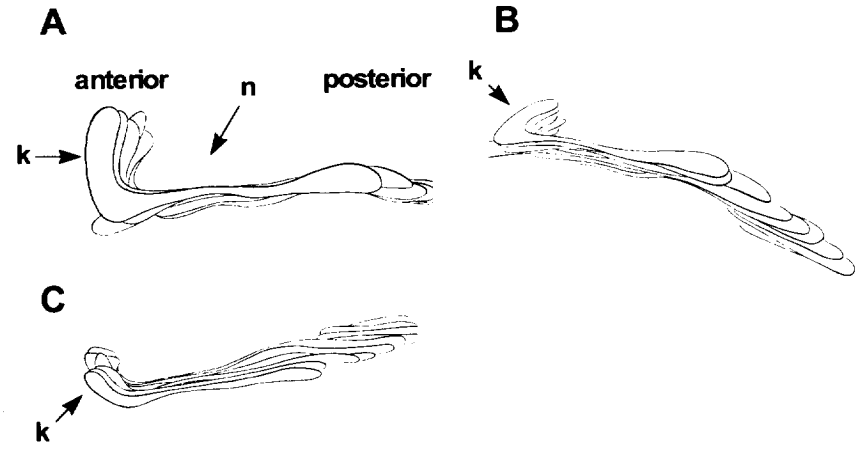

Fig. 2. Three dimensional reconstruction of the single mitochondrion in a non-dividing *L. tarentolae* cell. One set of serial sections was selected for this analysis. The contours of the outer membrane of the mitochondrion of the same cell in each section were traced onto cellulose acetate sheets, which were assembled to obtain the three dimensional reconstruction shown. A, the view of the reconstruction from above. B, C, artist drawings of stereo views of the same reconstruction from the sides, showing the extension of the mitochondrion into the base of the cell. The locations of the kinetoplast portion of the mitochondrion (k) and the nucleus (n) are indicated by arrows in A. The anterior and posterior directions of the cell are indicated in A.

3.3. Visualization of the single mitochondrion by immunofluorescence in stationary phase cells and dividing cells

An asymmetric mitochondrial structure which is remarkably similar to the electron microscopic reconstruction in Fig. 2 can be visualized in stationary phase cells by immunofluorescence, using a polyclonal antiserum to an 18 kD mitochondrial protein, as shown in Fig. 3A [18]. The mitochondrial structure stained by the p18 antiserum is also visible in the paraformaldehyde-fixed, Triton-extracted cells as a phase-dense structure in Fig. 3A-1.

p18 has been previously shown by cell fractionation experiments to be mitochondrial-localized by its presence in a highly purified mitochondrial cell fraction and its absence in a cytosolic fraction, and a localization of p18 in the mitochondrial inner membrane was suggested by the retention of the protein in a membrane fraction after Triton lysis and clarification of the mitochondrial fraction. The p18 gene also contains 17 N-terminal amino acids not present in the mature protein, which probably represent a mitochondrial targeting sequence. Consistent with these data, the p18 protein has recently been identified as a homologue of the mitochondrial ATPase subunit B (R. Benne and P. Sloof, personal communication).

A similar asymmetric mitochondrial morphology in long, slender stationary phase cells can be visualized by staining cells in vivo with the mitochondrial-specific dye, rhodamine 123 [23,24]. The stained cells are dried and fixed on a slide for observation in a UV microscope, as shown in Fig. 4A.

In the shorter rounded dividing cells, which contain a single kinetoplast and a single nucleus and therefore are located in the G1, S or early G2 phase of the cell cycle, a quite different mitochondrial structure is visualized both by immunofluorescence using the p18 antiserum and by in vivo staining with rhodamine 123. As shown in the p18-immunoflorescence images in Fig. 3B and the rhodamine-stained images in Fig. 4B, the mitochondrion extends from both sides of the kinetoplast region and appears to be joined in the posterior region of the cell. In some cells, the stained mitochondrion extends as a complex structure into the posterior of the cell.

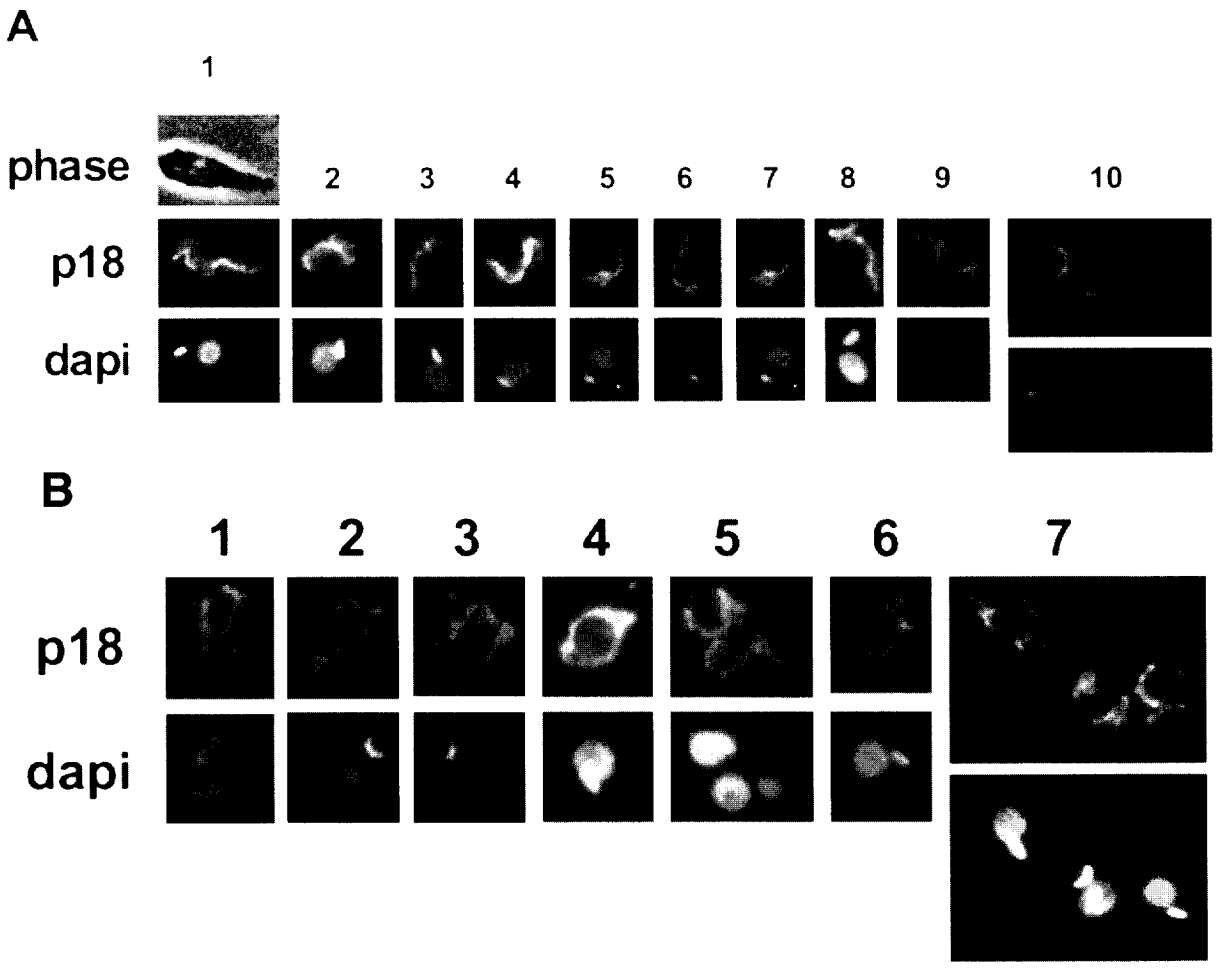

Fig. 3. Visualization of the single mitochondrion in *L. tarentolae* cells by immunofluorescence using antiserum against the mitochondrial membrane-specific p18 protein. A, non-dividing cells. The same cell is shown stained with the DNA-staining dye, DAPI, and with p18-antiserum. A phase contrast micrograph is shown in 1. The kDNA nucleoid body can be seen as a brightly stained elongated body in the DAPI-stained images. B, dividing cells showing the symmetric circular mitochondrion. In panel 5, the kinetoplast of the lower cell is obscured by the nucleus, and the second nucleus at the lower right is actually from another cell.

An electron microscopic serial section reconstruction of the mitochondrion in these cells was not performed, but it is clear from the immunofluorescence and rhodamine-staining results in Fig. 3B and Fig. 4B, that the symmetric three dimensional organization of the mitochondrion is quite different from the asymmetric organization seen in the non-dividing cells in Fig. 3A and Fig. 4A. The possibility exists that the mitochondrion in the dividing cells is actually cup- or sphere-shaped extending over the nucleus, but this is made unlikely, although not excluded, by the fact that stationary phase cells have a single mitochondrial tubule extending from one end of the kinetoplast, rather than the cup-shaped structure which would be predicted to result from division of the former type of mitochondrion. A high resolution confocal microscopy analysis may resolve this issue, but this remains to be done.

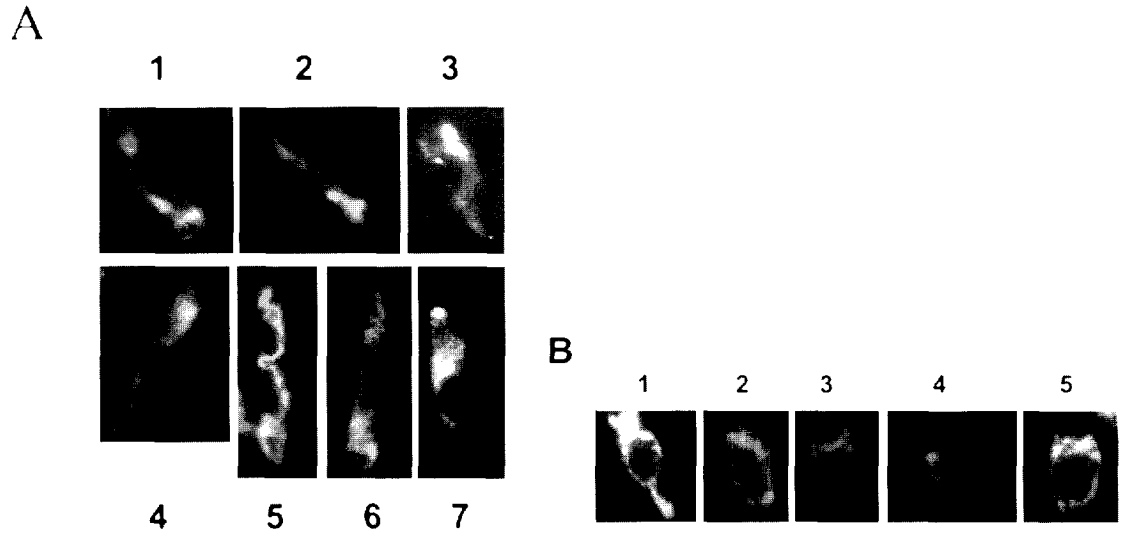

Fig. 4. Staining of the mitochondrion with rhodamine 123. A, asymmetric mitochondrion in long, slender non-dividing cells. B, circular, symmetric mitochondrion in short, rounded dividing cells.

4. Discussion

The use of the p18 antiserum as a marker for the entire mitochondrial membrane system in these cells is justified by the previously demonstrated mitochondrial localization of this component of the F1-F0 ATPase complex [18]. In vivo staining of cells with rhodamine 123 showed similar mitochondrial morphologies. The coincidence of the images of the asymmetric mitochondrion visualized with both the p18 antiserum and rhodamine 123 staining and the three dimensional electron microscopic reconstruction provides additional evidence that the p18 antiserum recognizes the entire mitochondrion and not a selected portion.

We propose that the observed asymmetric configuration of the single mitochondrion in elongated non-dividing *L. tarentolae* cells in culture is a direct result of the division of the kinetoplast portion of the circular symmetric mitochondrion during cell division, as diagrammed in the model in Fig. 5. Each daughter cell receives half of the kinetoplast and its DNA network as well as one of the mitochondrial tubular arms extending into the posterior of the cell. We speculate that in dividing cells, the mitochondrion then becomes circular either by growth of the mitochondrial tubule from the base to the kinetoplast or by growth from the kinetoplast to the base and fusion at the base. The resulting cell with a symmetrically organized mitochondrion is then prepared for another cell division, which results again in an equal but asymmetric distribution of mitochondrial membranes to the daughter cells. The single mitochondrion in other kinetoplastid species, such as log phase procyclic *Trypanosoma brucei* [25,26], *C. fasciculata*, *B. culicis* and epimastigote *T. cruzi* [4–6], is much more complex than that observed in *L. tarentolae*, often consisting of a symmetric reticulum of interconnected tubules. In addition, the presence of an asymmetric mitochondrial tubule has been reported in trypomastigotes of *T. cruzi* [4], which are normally non-dividing cells. Paulin, in fact, speculated that the functional significance of the symmetric mitochondrion is to insure equal distribution of mitochondrial elements during division [5], but apparently did not appreciate the significance of the asymmetric mitochondrion in *T. cruzi* trypomastigotes. A progression between a circular symmetric mitochondrion and an asymmetric mitochondrion probably also occurs during the cell cycle of these and other kinetoplastid protozoa, just as in the

case of *L. tarentolae*, but this remains to be examined.

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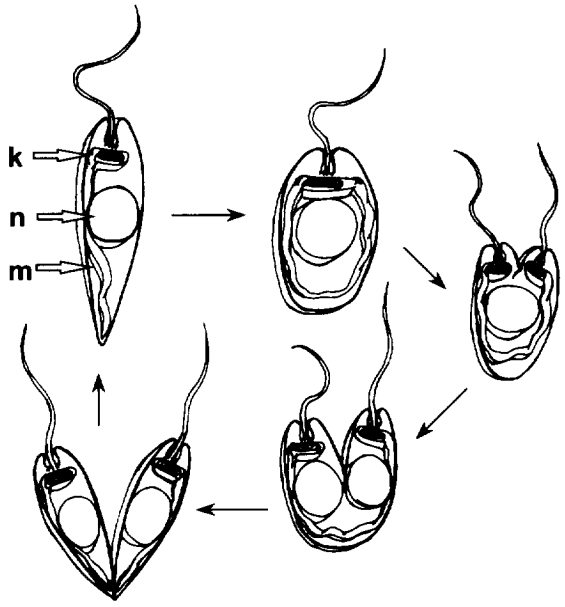

Fig. 5. Diagrammatic representation of the division of a *L. tarentolae* cell with a proposed mitochondrial division and biogenesis scheme based on the data in this communication. k, kinetoplast portion of the mitochondrion; n, nucleus; m, mitochondrial tubule.

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