NOTE

Mitosomes in Trophozoites and Cysts of the Reptilian Parasite Entamoeba invadens

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Heat shock protein genes led to the discovery of mitosomes in Entamoeba histolytica, but mitosomes have not been described for any other Entamoeba species, nor have they been identified in the cyst stage. Here, we show that the distantly related reptilian pathogen Entamoeba invadens contains mitosomes, in both trophozoites and cysts, suggesting all Entamoeba species contain these organelles.

Mitochondria have played a crucial role during eukaryotic evolution. They enabled and facilitated the development of multicellular life by relaxing the energetic constraints facing prokaryotes (11). The discovery of genes encoding proteins that are normally targeted to mitochondria in other eukaryotes (3) in the genome of Entamoeba histolytica, an organism previously thought to represent an earlier phase of eukaryotic life, raised doubts about the presumed premitochondrial status of this intestinal parasite. Antibodies raised against one of these, the mitochondrial chaperonin Hsp60, clearly showed the presence of an organelle, which was called a mitosome (21) or crypton (13). When the mitochondrial leader sequence of Hsp60 was removed, the protein accumulated in the cytosol, a phenotype that could be reversed by replacing the presequence with a genuine mitochondrial targeting sequence from another species, suggesting that the discovered organelle was indeed mitochondrial in nature (21).

Although mitosomes have been discovered in other former Archezoa (22, 25), the function of these organelles is not obvious. Analyses of the genomes of these human pathogens only suggested a handful of genes whose products are targeted to mitosomes. Most of these are “structural” in nature and encode heat shock proteins and metabolite or protein importers. Only a few “functional” enzymes have been discovered, and those involved in iron-sulfur cluster assembly seem to be a common denominator for all mitosomes. Unexpectedly, these proteins of clear mitochondrial ancestry have been replaced by lateral gene transfer with a much simpler system in E. histolytica (1, 24). Whether these proteins are genuinely mitosomal in this organism remains a matter of dispute (14, 16). The most

FIG. 1. Analyses of the amino-terminal regions of the mitochondrial chaperones Hsp60 (A) and mHsp70 (B) from the Amoebozoa. The bacterial homologue from Rickettsia prowazekii is shown for comparison. Acanthamoeba castellanii Hsp60 is not shown due to being incomplete at the N terminus. Predicted or confirmed cleavage sites are indicated by a dash, while the asterisk denotes an incomplete N terminus. The underlined residues for E. histolytica Hsp60 have been shown to be required for mitosomal targeting (21).
thorough attempt to understand mitosomal function in *E. histolytica* employed mass spectroscopy on Percoll-purified mitosomes (16). Frustratingly, two-thirds of the 95 identified proteins were hypothetical proteins. However, this study indicated that *E. histolytica* mitosomes are involved in sulfate activation (16), a thus far unique mitosomal trait of the *Entamoeba* organelles.

There are two stages in the *E. histolytica* life cycle: a motile trophozoite stage found inside the human host and a resistant infectious cyst which is excreted by infected individuals. The factors controlling encystation and excystation in *E. histolytica* are poorly understood, and our lack of knowledge is further hampered by our inability to induce cyst formation in vitro. As a consequence, nothing is known about what happens to mitosomes in cysts. The reptilian pathogen *Entamoeba invadens* acts as a proxy for the study of cyst formation as it is relatively straightforward to induce encystation in this *Entamoeba* species (18). As mitosomes have never been identified in any other member of the genus *Entamoeba* and there is no knowledge regarding the fate of mitosomes in cysts, we have studied the distribution of mitosomes in the reptilian pathogen *Entamoeba invadens*.

One of us previously demonstrated that the mitochondrial-type Hsp70 (mHsp70) chaperone is enriched in mitosomal fractions (20). In order to validate that work, which was performed using a heterologous mHsp70 antibody (23), we obtained a homologous antibody raised against recombinant *E. histolytica* mitosomal Hsp70. To maximize recombinant protein production, codon usage was converted from *E. histolytica* to *Escherichia coli* using JCat (7). Subsequently, a synthetic gene was constructed, which included a C-terminal histidine tag for protein purification and the restriction site BamHI at both termini to enable cloning into the BamHI site of the pET-3c expression vector. The poly-His-tagged recombinant protein was produced in *Escherichia coli* BL21(DE3)(pLysS) cells and purified under nondenaturing conditions by immobilized-metal ion-affinity chromatography using Ni-nitrilotriacetic acid (NTA). Correct protein identity was verified by mass spectroscopy, and this protein was subsequently used for immunization.

*E. histolytica* HM-1:IMSS and *E. invadens* IP-1 were grown using standard conditions. Cyst formation was induced according to established protocols (18). For *E. invadens* trophozoite localization experiments, cells were washed in phosphate-buffered saline (PBS) and fixed with 3% formaldehyde in PBS for 45 min while mature cysts were fixed using 3% formaldehyde in PBS overnight at 4°C. The dehydrated specimens were...
rehydrated with PBS for 30 min, permeabilized with 0.2% Triton X-100 in PBS for 20 min at room temperature, and blocked for 2 h with 2% bovine serum albumin (BSA) in PBS. The cell preparations were incubated with titrated *E. histolytica* Hsp60 (1:100) (a kind gift of C. Graham Clark) and *E. histolytica* mHsp70 (1:100) (this study) antibodies in PBS with 2% BSA and 0.2% Triton X-100 for 1 h at room temperature in a humid chamber. Secondary antibodies coupled to Alexa Fluor 594 and 488 (Invitrogen), respectively, were used to detect bound antibodies. Specimens were thoroughly washed in PBS with 0.5% BSA and 0.05% Triton X-100 between incubations and finally embedded with Vectashield (Vector Labs) or Glycergel (Dako) mounting medium. Nuclear DNA was detected with the intercalating agent 4',6-diamidino-2-phenylindole (DAPI). Immunofluorescence image data collection was performed on a Leica SP2 AOBS confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany) with an oil immersion objective (Leica, HCX PL APO 63 x 1.4) and a pinhole setting of Airy 1 with 2-fold oversampling. Image stacks were further processed using the Huygens deconvolution software package, version 2.7 (Scientific Volume Imaging, Hilversum, Netherlands). Three-dimensional reconstruction, volume rendering, and colocalization analysis were done with the Imaris software suite, version 7.2 (Bitplane, Zurich, Switzerland).

In order to understand the phylogenetic relationship of amoebozoan mHsp70, a data set of 39 protein sequences with 25 eukaryotic and 14 prokaryotic taxa was assembled. Protein sequences were aligned using ClustalW in SeaView version 4.2.12. The data set contained 620 informative patterns from a total of 704 sites. Phylogenies were calculated using the model-based maximum likelihood approach (ML) using PhyML (8) and the Bayesian inference approach using MrBayes (17). For ML analyses, modelgenerator v.0.85 (10) suggested the model LG+F, with 8 rate categories and an alpha shape parameter of 0.48 to fit the observed data best. Four Bayesian analyses were run using a mixed-amino-acid model accommodating 4 rate+inv categories containing 4 chains each. One million generations were calculated, and trees were sampled every 1,000 generations. The model stabilized rapidly, and 250 trees were discarded as burn-in. Mitochondrial targeting signals were analyzed using the localization prediction tools WoLF PSORT (9) and Mitoprot (4).

The presence of mitosomes in *E. histolytica* is well documented,
but there is no information regarding the presence of these organelles for any other *Entamoeba* species. As there is (partial) genome information available for several other *Entamoeba* species, we decided to screen these genomes for the presence of Hsp60 and mHsp70. Putative Hsp60 and mHsp70 sequences were identified in *Entamoeba dispers* and *E. invadens*, but incomplete Hsp60 sequences lacking their N termini could only be identified for *Entamoeba terrapinae* and *Entamoeba moshkovskii*, while no mHsp70 sequences could be identified with reasonable certainty in these two species. As shown before (6), targeting signal prediction programs have difficulties identifying *Entamoeba* mitochondrial precursors, but alignment of the N termini of Hsp60 and mHsp70 clearly indicates the presence of precursors that are upstream of the analogous prokaryotic N terminus and which have been shown to be genuine targeting signals in *E. histolytica* (Fig. 1).

In order to confirm the mitochondrial nature of the identified putative mHsp70, we conducted phylogenetic analyses, including extended amoebozoan sampling (Fig. 2). Our analyses clearly confirm the mitochondrial ancestry and monophyly of amoebozoan mHsp70s. Although we correctly recovered the sister group relationship with the opisthokonts, this node was only weakly supported. As shown previously (2), alphaproteobacteria are basal to all eukaryotes in accordance with their supposed role as donor of the mitochondrial endosymbiont.

To verify our *in silico* analyses, which clearly predict the *Entamoeba* mHsp70 to be mitochondrial, we conducted laser-scanning confocal microscopy and three-dimensional image rendering using the homologous Hsp60 and mHsp70 antisera on *E. histolytica* whole-cell preparations. The Hsp60 antibody localizes in a discrete punctate pattern and abundance, similar to previous reports (12, 16), and the homologous mHsp70 antiserum colocalizes to the same areas (Fig. 3A to D), confirming the earlier fractionation data (20). When these antisera were used on the distantly related *E. invadens*, a similar localization pattern was observed. However, as the representative images in Fig. 3 and 4 show, we regularly detected at least 10-fold fewer organelles in *E. invadens*. This clearly suggests that this distant *Entamoeba* species (19) contains mitosomes, as well allowing us to suggest all species in the genus do contain this organelle. Although perhaps discounted by most, some still entertain the possibility of genuine extant Archezoa with this organelle. Although perhaps discounted by most, some still allow us to suggest all species in the genus do contain this organelle. We also show that mitosomes are abundant in the infectious cysts, suggesting that these enigmatic organelles may play a role in this important life cycle stage as well.

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REFERENCES

1. Ali, V., Y. Shigeta, U. Tokumoto, Y. Takahashi, and T. Nozaki. 2004. An intestinal parasitic protozoon, *Entamoeba histolytica*, possesses a non-redundant nitrogen fixation-like system for iron-sulphur cluster assembly under anaerobic conditions. J. Biol. Chem. 279:16863–16874.
2. Bakatselou, C., C. Kigzell, and C. G. Clark. 2000. A mitochondrion-type hsp70 gene of *Entamoeba histolytica*. Mol. Biochem. Parasitol. 110:177–182.
3. Clark, C. G., and A. J. Roger. 1995. Direct evidence for secondary loss of mitochondria in *Entamoeba histolytica*. Proc. Natl. Acad. Sci. U. S. A. 92: 6518–6521.
4. Claros, M. G., and P. Vences. 1996. Computational method to predict mitochrondially imported proteins and their targeting sequences. Eur. J. Biochem. 241:779–786.
5. de Duve, C. 2007. The origin of eukaryotes: a reappraisal. Nat. Rev. Genet. 8:593–606.
6. Dolezal, P., et al. 2010. The essentials of protein import in the degenerate mitochondrion of *Entamoeba histolytica*. PLoS Pathog. 6:e1000812.
7. Grote, A., et al. 2005. Jcat: a novel tool to adapt codon usage of a target gene to its potential expression host. Nucleic Acids Res. 33:W526–W531.
8. Guindon, S., and T. Gascuel. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies. Syst. Biol. 52:696–704.
9. Horton, P., et al. 2007. WoLF PSORT: protein localization predictor. Nucleic Acids Res. 35:W585–W587.
10. Keane, T., C. Creevey, M. Pentony, T. Naughton, and J. McInerney. 2006. Assessment of methods for amino acid matrix selection and their use on empirical data shows that ad hoc assumptions for choice of matrix are not justified. BMC Evol. Biol. 6:29.
11. Lane, N., and W. Martin. 2010. The energetics of genome complexity. Nature 467:929–934.
12. León-Avila, G., and J. Tovar. 2004. Mitosomes of *Entamoeba histolytica* are abundant mitochondrion-related remnant organelles that lack a detectable organelar genome. Microbiology 150:1245–1250.
13. Mai, Z., et al. 1999. Hsp60 is targeted to a cryptic mitochondrion-derived organelle (“crypton”) in the microaerophilic protozoan parasite *Entamoeba histolytica*. Mol. Cell. Biol. 19:2198–2205.
14. Maralikova, B., et al. 2010. Bacterial-type oxygen detoxification and iron-sulphur cluster assembly in amoebal relict mitochondria. Cell. Microbiol. 12:331–342.
15. Margulis, L., M. Chapman, and M. F. Dolan. 2007. Schemes for analysis of evolution: de Duve’s peroxisomes and Meyer’s hydrogenases in the sulphur-oxidizing Proteotrocoy taxon. Nat. Rev. Genet. 8:1–2.
16. Mi-ichi, F., M. Abu Yousuf, K. Nakada-Tsukui, and T. Nozaki. 2009. Mitosomes in *Entamoeba histolytica* contain a sulfate activation pathway. Proc. Natl. Acad. Sci. U. S. A. 106:21731–21736.
17. Ronquist, F., and J. P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574.
18. Sánchez, L., V. Enea, and D. Eichinger. 1994. Identification of a developmentally regulated transcript expressed during encystation of *Entamoeba invadens*. Mol. Biochem. Parasitol. 67:125–135.
19. Stensvold, C. R., et al. 2011. Increased sampling reveals novel lineages of *Entamoeba* consequences of genetic diversity and host specificity for taxonomy and molecular detection. Protist 162:525–541.
20. Tovar, J., S. S. E. Cos, and M. van der Giezen. 2007. A mitochondrion paraffold based on Peroxil density gradients and its use in validating the mitosomal nature of *Entamoeba histolytica* mitochondrial Hsp70. Methods Mol. Biol. 390:167–177.
21. Tovar, J., A. Fischer, and C. G. Clark. 1999. The mitosome, a novel organelle related to mitochondria in the amitochondrial parasite *Entamoeba histo- lytica*. Mol. Microbiol. 32:1013–1021.
22. Tovar, J., et al. 2003. Mitochondrial remnant organelles of *Giardia* function in iron-sulphur protein maturation. Nature 426:172–176.
23. van der Giezen, M., et al. 2003. Fungal hydrogenosomes contain mitochondrial heat-shock proteins. Mol. Biol. Evol. 20:1051–1061.
24. van der Giezen, M., S. Cox, and J. Tovar. 2004. The iron-sulphur cluster assembly genes iscS and iscU of *Entamoeba histolytica* were acquired by horizontal gene transfer. BMC Evol. Biol. 4:67.