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The mitosome, a novel organelle related to mitochondria in the amitochondrial parasite *Entamoeba histolytica*

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Summary

Ultrastructural analysis of *Entamoeba histolytica* reveals that this intestinal human pathogen lacks recognizable mitochondria, but the presence in its genome of genes encoding proteins of mitochondrial origin suggests the existence of a mitochondrially derived compartment. We have cloned the full-length *E. histolytica* gene encoding one such protein, chaperonin CPN60, and have characterized its structure and expression. Using an affinity-purified antibody raised against recombinant protein, we have localized native *E. histolytica* CPN60 to a previously undescribed organelle of putative mitochondrial origin, the mitosome. Most cells contain only one mitosome, as determined by immunofluorescence studies. *Entamoeba histolytica* CPN60 has an amino-terminal extension reminiscent of known mitochondrial and hydrogenosomal targeting signals. Deletion of the first 15 amino acids of CPN60 leads to an accumulation of the truncated protein in the cytoplasm. However, this mutant phenotype can be reversed by replacement of the deleted amino acids with a mitochondrial targeting signal from *Trypanosoma cruzi* HSP70. The observed functional conservation between mitochondrial import in trypanosomes and mitosome import in *Entamoeba* is strong evidence that the *E. histolytica* organelle housing chaperonin CPN60 represents a mitochondrial remnant.

Introduction

Entamoeba histolytica, an intestinal parasite of humans, was for many years used to illustrate the probable structure of an ancestral eukaryote (Meza, 1992; Bakker-Grunwald and Wöstmann, 1993). Electron microscopy revealed the absence of many characteristic organelles of eukaryotes – mitochondria, Golgi apparatus, rough endoplasmic reticulum, microtubular cytoskeleton (Martinez-Palomo, 1986) – and biochemical analyses showed that it lacked glutathione and its associated biosynthetic enzymes (Fahey *et al*., 1984). These findings were interpreted as indicating the primitive nature of the organism. However, with the advent of ribosomal RNA-based molecular phylogenies, a different picture emerged. In these analyses *E. histolytica* diverged from other eukaryotes much later than lineages that contained all of the missing organelles (Sogin *et al*., 1989), implying that their absence from *Entamoeba* was due to secondary loss.

In *E. histolytica*, direct evidence for secondary loss of mitochondria was recently obtained (Clark and Roger, 1995) by the identification in its nuclear genome of two genes encoding proteins that, in other eukaryotes, reside in the mitochondrion: the enzyme pyridine nucleotide transhydrogenase (PNT) and the mitochondrial chaperonin CPN60. Both *E. histolytica* proteins are predicted to have amino-terminal extensions reminiscent of organelle targeting sequences, and phylogenetic analyses strongly support a mitochondrial origin for CPN60 (Clark and Roger, 1995; Roger *et al*., 1998). Although *E. histolytica* PNT enzyme activity is sedimentable (Harlow *et al*., 1976), perhaps indicating association with an intracellular compartment, the location of CPN60 has not been determined.

In this report, we have analysed the genomic arrangement of the *E. histolytica* cpn60 gene and its expression. We have raised antibodies against recombinant CPN60 protein and present evidence for the existence of a cellular compartment, the mitosome, to which the *E. histolytica* CPN60 is targeted. We have also examined the role of the amino-terminal residues of CPN60 for protein import into the mitosome and observed a conservation of function between the CPN60 leader sequence and an authentic mitochondrial targeting signal, further evidence supporting the mitochondrial origin of this novel *E. histolytica* organelle.

Results

Sequence analysis of the E. histolytica *cpn60 gene*

A full-length cpn60 gene sequence, assembled as described in *Experimental procedures*, predicts a protein of

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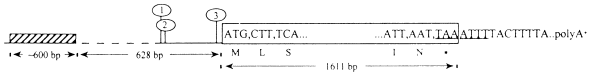

Fig. 1. Structure of the *E. histolytica* cpn60 gene. The coding region of the gene, represented by nucleotide triplets encoding the first three and the last two amino acid residues of CPN60, is boxed. The nucleotide sequence of the cpn60 coding region predicts a protein of 536 amino acids, 56.8 kDa, with an isoelectric point of 8.3. The 12 nucleotides immediately downstream of the coding region and preceding the polyadenylated tail are shown. The putative polyadenylation signal, which overlaps the translation stop codon, is underlined. Upstream of the coding region, the cross-hatched box represents the repetitive element homologous to that described by Mittal *et al*. (1994). Circled numerals 1, 2 and 3 represent the TATA box, the GAAC box and the initiator element, respectively, as described in the text. The complete nucleotide sequence represented in the figure can be obtained from databanks under accession number AF029366.

≈57 kilodaltons with a basic isoelectric point (Fig. 1). DNA blot analysis suggests that the cpn60 gene is present as a single copy per haploid genome (not shown). Analysis of the 1.2 kb of DNA upstream of the coding region identified no significant open reading frames on either strand, but comparison with DNA sequences in databases revealed significant similarity to a repetitive DNA sequence from *E. histolytica* as previously described (Mittal *et al*., 1994). This sequence, HMc, was found to be dispersed in the genome, present in 25–30 copies per cell, lacking open reading frames and was not transcribed into a stable RNA. The copy upstream of the *E. histolytica* cpn60 gene has ≈86% sequence identity to HMc over a span of almost 600 base-pairs. The original HMc element is located upstream of an open reading frame encoding an unidentified protein. It is interesting to note that the start codon of that protein is 592 bp downstream of the element, whereas that of cpn60 is 628 bp downstream (Fig. 1). Both elements are also in the same orientation with respect to the coding region. Their biological significance is currently unknown.

A number of conserved sequence motifs have been identified 5′ to the start codons of *E. histolytica* genes, and some of these have been shown to have characteristics of promoter elements (Buss *et al*., 1995; Purdy *et al*., 1996). Examination of the sequences adjacent to the cpn60 coding region has revealed similar motifs (Fig. 1). The presumptive initiator element has a consensus of 5′-AAAA-ATTCA-3′ and is usually found between positions −1 to −21 with respect to the start codon. The sequence 5′-TAACAATCA-3′ is found at −13 to −5 from the cpn60 start codon and is most similar to variant CE1a as defined previously by Purdy *et al*. (1996). The presumptive 'TATA box' in *E. histolytica* has the consensus sequence 5′-GTATTTAAAG/C-3′ and is usually located between −52 and −28 relative to the start codon of the gene. The cpn60 locus has the sequence 5′-TTGTTGAAAG-3′ at positions −36 to −27 from the start codon. A closer match 5′-ATATTTAAAA-3′ can be found at around position −170, but this would be well outside the normal position seen for this element. Some *E. histolytica* genes have a third consensus element, the 'GAAC' box, with a consensus of 5′- AAAGAACT-3′ located between the initiator and TATA box consensus elements (Singh *et al*., 1997). The closest match adjacent to the cpn60 gene is 5′-AAA-GAATT-3′ at positions −31 to −24, which overlaps with the TATA box-like sequence identified above. A conserved sequence 5′-TAA/TTT-3′ that occurs 5–14 bases upstream from the mRNA polyA tail has been suggested as a putative polyadenylation signal (Bruchhaus *et al*., 1993). The *E. histolytica* cpn60 gene contains, at its 3′ terminus, the sequence 5′-TAAATTT-3′, −14 to −8 nucleotides preceding the polyadenylation site. Notably, this putative polyadenylation signal overlaps the translation termination codon (Fig. 1).

Expression of the cpn60 gene

The presence of a full-length cpn60 coding sequence preceded by typical *E. histolytica* expression signals and the detection of cpn60-specific polyadenylated mRNA by RT-PCR suggested a functional gene. Its expression was investigated in cultured trophozoites by Northern and Western blotting using an internal cpn60 DNA fragment and an affinity-purified anti-CPN60 antibody (see *Experimental procedures*) as probes. Northern blot analysis revealed the presence of a single transcript of about 1.65 kb, in good agreement with the size predicted from the DNA sequence (Fig. 2A). Western blotting confirmed the synthesis of CPN60 in trophozoites. A major peptide band of ≈57 kDa was detected in blots, in agreement with the molecular size predicted for the *E. histolytica* protein (Fig. 2B). Additional, smaller bands were also highlighted by the antibody in some extracts analysed (see for example Fig. 4). These are most probably the result of residual proteolytic degradation during sample processing, despite the use of protease inhibitors, as *E. histolytica* is known to contain a wide variety of highly active proteases (Martinez-Palomo, 1986).

Expression in response to cellular stresses

In mammalian cells and many other organisms, chaperonin-encoding genes are transcriptionally activated upon heat shock and other environmental stresses (Linquist and Craig, 1988). We therefore examined whether the *E. histolytica* cpn60 gene was subject to a similar type of regulation by incubating cells at 41°C, an elevated temperature known to cause stress in this organism (Gillin and Diamond, 1980). Figure 3 shows that the cpn60 gene is not activated by heat shock, as determined by comparison of steady-state cpn60 transcript levels with those of

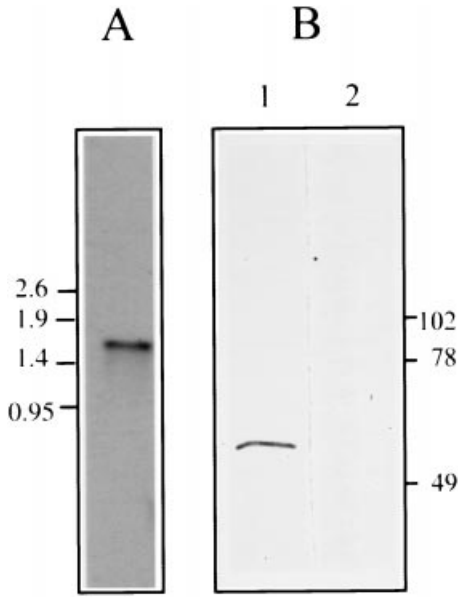

Fig. 2. Expression of *E. histolytica* CPN60.
A. mRNA (0.5 μg) isolated from trophozoites was analysed by Northern blotting as described in *Experimental procedures*. The blot was probed with a PCR-amplified fragment of cpn60 (Clark and Roger, 1995). Molecular sizes of RNA markers (Promega) are given in kilobases.
B. Western blot analysis of trophozoite protein extracts separated in a 7.5% polyacrylamide gel and blotted onto nitrocellulose. Membrane strips were incubated with 1:100 dilution of an affinity-purified anti-*E. histolytica* CPN60 antibody (lane 1) or with an equivalent dilution of preimmune rabbit serum (lane 2). Sizes of prestained protein markers (low-range, Bio-Rad) are given in kilodaltons.

the constitutively expressed β-actin gene and by monitoring CPN60 protein levels by Western blotting in a time course experiment. Furthermore, no induction of expression was observed upon exposure to other stressful environmental conditions such as increased oxygen tension or ethanol concentrations as high as 3% (data not shown), suggesting that *E. histolytica* CPN60 and its encoding gene are not subject to regulation by general stress under the conditions used. These results are in contrast to the tight stress regulation observed for other CPN60 homologues but in agreement with the observation that in *Giardia lamblia*, another amitochondrial human pathogen, the expression of CPN60 is not responsive to stress (Roger *et al*., 1998).

Cellular localization of the E. histolytica *CPN60 protein*

Mitochondrial CPN60, in conjunction with its co-protein CPN10, is known to prevent aggregation of cellular proteins imported into the organelle through the formation of a multisubunit complex that aids in the ATP-dependent folding of a wide range of proteins (Horwich *et al*., 1993; Ewalt *et al*., 1997). Although in amitochondrial *E. histolytica* its cellular localization had not been determined, its physiological function in other eukaryotes suggested that it might be targeted to an as yet unidentified subcellular organelle. We fractionated *E. histolytica* cell extracts by differential centrifugation into nuclear, 'mitochondrial', heavy microsomal, light microsomal and cytoplasmic fractions as described in *Experimental procedures* and monitored the presence of CPN60 by Western blotting. A clear enrichment of CPN60 was observed in the heavy microsomal and light microsomal fractions (Fig. 4A), demonstrating an association of CPN60 with cellular microbodies. Furthermore, using a protease protection assay, we were able to demonstrate that *E. histolytica* CPN60 is indeed located inside a membrane-bound organelle as indicated by its sensitivity to trypsin degradation in the presence, but not in the absence, of a permeabilizing agent (Fig. 4B).

Initial attempts to localize CPN60 by immunofluorescence using the anti-CPN60 antibody were unsuccessful, apparently because of the antibody only recognizing denatured

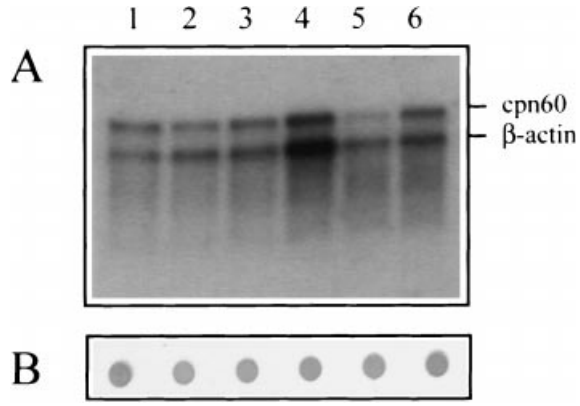

Fig. 3. Expression of *E. histolytica* CPN60 in response to heat shock.
A. Northern blot analysis of mRNA from trophozoites exposed to 41°C for 0 h, lane 1; 0.5 h, lane 2; 1 h, lane 3; 2 h, lane 4; 4 h, lane 5; and 6 h, lane 6. The blot was probed with a PCR-amplified fragment of the β-actin gene and exposed to film. After removal of the probe by a 30 min incubation in a boiling solution of 0.1% SDS, the membrane was immediately reprobed with an internal fragment of cpn60 and exposed to X-ray film. Using this method it was possible to compare, on the same blot, transcript levels of cpn60 and of the constitutively expressed β-actin gene. Variations in the amount of cpn60 mRNA in the time course experiment are mimicked by changes in the amount of β-actin mRNA, indicating that these changes are due to loading differences between lanes and are not a reflection of induced changes in steady-state transcript levels. The pattern generated by the residual radioactivity of β-actin above is identical to the pattern observed upon initial exposure of the blot probed only with β-actin (not shown). By monitoring original and residual radioactivity, it is estimated that the β-actin mRNA copy number is at least 20 times higher than that of cpn60. The identities of transcript bands are indicated on the right.
B. Western blot analysis of protein extracts of trophozoites exposed to heat shock as above. Protein aliquots (20 μg) were dot-blotted onto nitrocellulose strips and probed with the affinity-purified anti-*E. histolytica* CPN60 antibody as described in *Experimental procedures*.

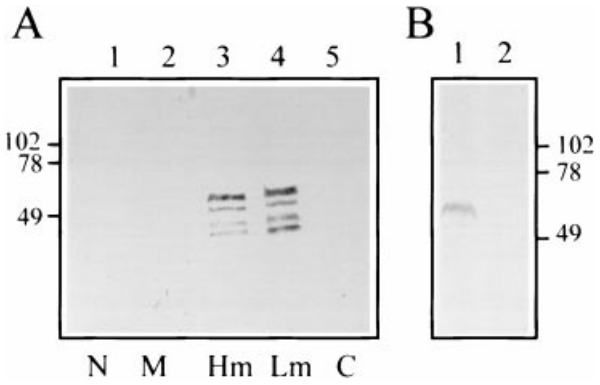

Fig. 4. Association of *E. histolytica* CPN60 with cellular microsomes.
A. Cell-free extracts were fractionated by differential centrifugation as described in *Experimental procedures*. Aliquots of these fractions (40 μg) were separated by electrophoresis in a 7.5% SDS–PAGE gel, electroblotted onto nitrocellulose and probed with an affinity-purified anti-*E. histolytica* CPN60 antibody. Indicated fractions are N, nuclear; M, 'mitochondrial'; Hm, heavy microsomal; Lm, light microsomal; C, cytoplasmic. Partial proteolytic degradation of CPN60 can be observed.
B. Protection of CPN60 from trypsin degradation. Microsomes were prepared as above and treated with trypsin or trypsin plus Triton X-100 as described in *Experimental procedures*. Lane 1, microsomes treated with trypsin; lane 2, microsomes treated with trypsin and Triton X-100. Protein degradation observed in lane 2 was minimized by prior addition of trypsin inhibitor (not shown). Sizes of prestained protein markers (low-range, Bio-Rad) are given in kilodaltons.

protein. As an alternative approach we transfected *Entamoeba* with a recombinant vector expressing CPN60 tagged with a c-*myc* epitope at its carboxy-terminus. Cellular structures of ≈1–2 μm in diameter were identified by fluorescence microscopy using an antic-*myc* tag antibody (Fig. 5). The structure was detected in ≈50–60% of the cell population, possibly because of differential cell permeability or because not all cells expressing the drug resistance marker might be expressing the chaperonin gene simultaneously. In most organelle-containing cells a single structure was identified, but a few cells containing two and, rarely, three structures were also observed. These results demonstrate the presence in *E. histolytica* of a novel organelle, the mitosome, containing the mitochondrially related chaperonin CPN60.

Protein targeting into the mitosome.

All mitochondrial CPN60 homologues examined to date are encoded by nuclear genes and contain amino-terminal extensions required for import into the organelle. We have previously reported that the *E. histolytica* cpn60 and PNT genes predict proteins with N-terminal domains rich in hydroxylated and basic amino acids that resemble known mitochondrial and hydrogenosomal organelle targeting sequences (Clark and Roger, 1995; Roger *et al*., 1998). We therefore investigated the role of the CPN60 amino-terminal extension in mitosome protein import by deletion mutagenesis and, to test further the putative mitochondrial origin of the mitosome, we replaced its N-terminal sequence with a functional mitochondrial targeting signal from *Trypanosoma cruzi*, a trypanosomatid protozoon. The predicted amino-termini and molecular sizes of wild-type and recombinant proteins encoded by transfection constructs A, B, C and D are presented in Fig. 6A. These constructs were transfected into *E. histolytica* trophozoites and extracts of established recombinant lines were fractionated by differential centrifugation as before into microsomal and cytoplasmic fractions. The presence of CPN60 was monitored by Western blotting using the anti-*E. histolytica* CPN60 antibody. In both untransfected control cells and recombinant cells expressing wild-type protein (from construct A) CPN60 accumulates in the microsomal fraction (lanes 1 and 2, Fig. 6B) providing evidence that the addition of the c-*myc* tag does not alter its organellar localization. The importance of the amino-terminal extension for mitosome targeting in *E. histolytica* is indicated by the removal of the first 15 amino acids of the protein in cells harbouring construct B, which leads to a clear enrichment of recombinant CPN60 in the cytoplasmic fraction (lane 3, Fig. 6B). Importantly, the addition of a functional mitochondrial targeting signal to the truncated protein (recombinant cells carrying construct C) reverses this mutant phenotype, leading once again to the accumulation of CPN60 in the microsomal fraction (lane 4, Fig. 6B). Such reversion of mutant phenotype demonstrates a conservation of function between mitochondrial import in trypanosomes and mitosome import in *E. histolytica*.

Discussion

The identification of mitochondrial remnants in *E. histolytica* confirms that this organism is not a surviving relic

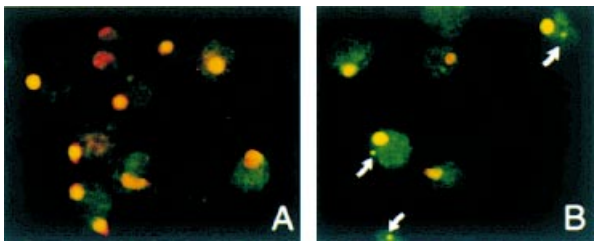

Fig. 5. Identification of the mitosome by cellular localization of *E. histolytica* CPN60 in trophozoites. Wild-type (A) and recombinant trophozoites expressing c-*myc*-tagged CPN60 (B) were fixed and permeabilized as described in *Experimental procedures* and probed with an anti-c-*myc* monoclonal antibody (Santa Cruz Biotechnology). Cells were finally stained with propidium iodide to identify the nuclei and examined by fluorescence microscopy. Clearly visible mitosomes are indicated by arrows.

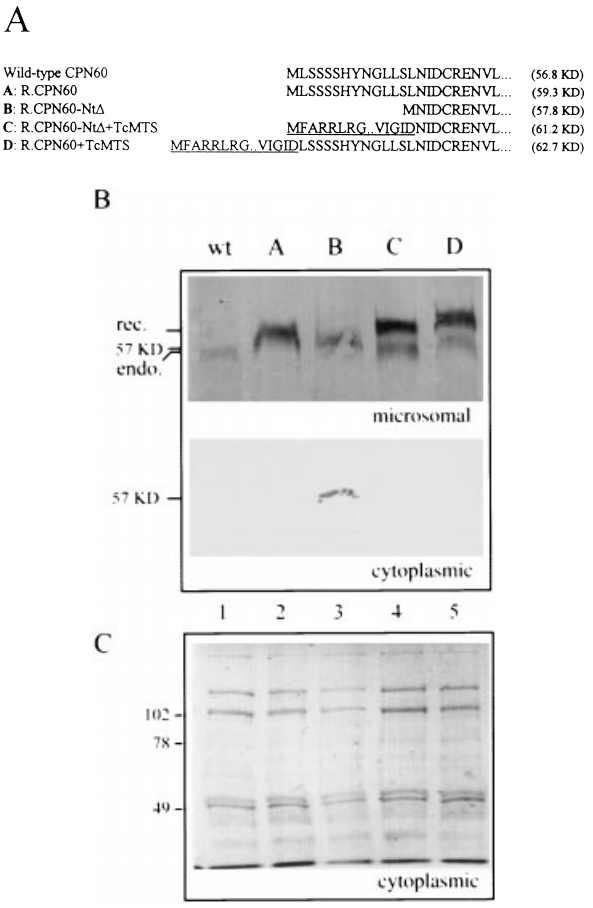

Fig. 6. Functional conservation between mitosomal and mitochondrial targeting signals.
A. Amino-terminal sequences and predicted molecular sizes of wild-type (top) and recombinant CPN60s expressed from recombinant constructs A, B, C, and D (*Experimental procedures*). NtΔ, amino-terminal deletion of the initial 15 residues; TcMTS, *T. cruzi* mitochondrial targeting signal. For convenience, only part of the mitochondrial targeting sequence is shown; the MTS is underlined.
B. Western blot analysis of proteins contained in the microsomal and cytoplasmic fractions. Cell-free extracts were fractionated by differential centrifugation and analysed by SDS–PAGE and Western blotting using a 1:100 dilution of an affinity-purified anti-*E. histolytica* CPN60 antibody. Lane 1, untransfected control (wt); lanes 2, 3, 4 and 5, fractions obtained from recombinant cells harbouring constructs A, B, C and D respectively. The identities of recombinant (rec.) and wild-type endogenous (endo.) CPN60, as determined by comparison with molecular size markers and with the untransfected control shown in lane 1, are indicated on the left.
C. Coomassie staining used to control for protein loading in the cytoplasmic fraction. The apparent migration of molecular size markers (Bio-Rad) is indicated.

of premitochondrial evolution. Instead, it is likely that the ancestors of *Entamoeba* once harboured mitochondria – or an endosymbiont related to the progenitor of mitochondria – and that a number of the organelle's physiological functions were lost secondarily, perhaps during adaptation to its current parasitic and mostly anaerobic way of life.

Mitochondrial and plastid genome derivation occurs widely in eukaryotes (Hanson and Folkerts, 1992; Wolstenholme, 1992; Egea and Lang-Unnasch, 1995; Gray *et al*., 1998). Mitochondrial genome derivation is particularly well documented in protists, a phylogenetically diverse group of mostly unicellular organisms with variable degrees of mitochondrial genome complexity, from the minimally derived *Reclinomonas americana* containing 69 kb of mtDNA (Lang *et al*., 1997) to the highly derived *Plasmodium* species *P. yeolii* and *P. falciparum* with 6 kb of mtDNA each (Feagin, 1994). The *E. histolytica* mitosome appears to have lost its entire genome, as no evidence for the presence of extranuclear DNA was obtained (Fig. 5). With one exception (Akhmanova *et al*., 1998) complete genome loss has also been observed in hydrogenosomes, energy-generating organelles of mitochondrial ancestry present in trichomonad flagellates and some fungi and rumen ciliates (Muller, 1993; Bui *et al*., 1996). In the absence of their own DNA, persistence of the mitosome in *Entamoeba* and the hydrogenosome in *Trichomonas* implies that proteins responsible for their biogenesis and replication must be encoded by nuclear genes, and that at least some of the organelles' physiological functions must be important for these organisms.

The amino-terminal extension of the *E. histolytica* chaperonin CPN60 is clearly important for mitosome targeting *in vivo*, as removal of the first 15 amino acids leads to an enrichment of the protein in the cytoplasm (Fig. 6B). Restoration of normal CPN60 organelle targeting after replacement of these 15 amino acids by a functional mitochondrial targeting signal from *T. cruzi* demonstrates a conservation of function between mitochondrial import in trypanosomes and protein import into the mitosome. Because conservation of function between trypanosomatid mitochondrial protein targeting and targeting of proteins into *T. vaginalis* hydrogenosomes has also been demonstrated (Hausler *et al*., 1997), it is likely that the *E. histolytica* mitosome shares a common ancestor with both mitochondria and hydrogenosomes.

According to the hydrogen hypothesis for the origin of the eukaryotic lineage (Martin and Muller, 1998), a type I amitochondriate eukaryote (organisms lacking compartmentalized energy-preserving mechanisms) such as *E. histolytica* could arise through secondary loss of organellar function either from a mitochondrial ancestor or from a type II amitochondriate eukaryote (organisms lacking respiration but retaining an energy-generating microbody, such as the hydrogenosome). The remarkable similarity between *E. histolytica* CPN60 and PNT organelle targeting signals and some hydrogenosomal targeting signals, particularly the abundance of hydroxylated amino acids, mostly serine, between residues 2–7 (Table 1), suggests the possibility that *E. histolytica* might have derived from a type II amitochondriate ancestor. This view is also

Table 1. Structural comparison of hydrogenosomal and mitosomal targeting signals.

Organism	Protein	Amino acid sequence	Reference
E. histolytica	PNT	MSTSSSIEEEVFNYMKIINNFVSVG ...	Clark and Roger (1995)
E. histolytica	CPN60	MLSSSSHYNGKLLSLNIDC**R**ENVLS ...	Roger *et al.* (1998)
T. vaginalis	β-Succinyl CoA synthetase 2	MLSSSFA**R**NFNILEWQSKEICAKYN ...	Lahti *et al.* (1992)
T. vaginalis	[Fe]-hydrogenase B	MLASSS**R**AMKGFAANIRWVDTSHNA ...	Bui and Johnson (1996)
T. vaginalis	Malic enzyme A	MLTSSVSVPV**R**NICRAKVPTLKTGM ...	Hrdy and Muller (1995)
T. vaginalis	Adenylate kinase	MLSTLAK**R**FASGKKDRMVVFFGPPG ...	Lange *et al.* (1994)
T. vaginalis	CPN60	MSLIEAAKHFT**R**AFAKARDLKFGSD ...	Bui *et al.* (1996)
T. cruzi	HSP70	MFARRLRGAGSLAAASLA**R**WQSSKV ...	Engman *et al.* (1992)

In all cases only the initial 25 amino acid residues are shown. Peptides known to be proteolitically removed from mature proteins are underlined. Arg residues known or predicted to locate at position −2 relative to the predicted cleavage sites are shown in bold type; the equivalent residue for PNT is Arg-37. The HSP70 mitochondrial targeting signal used in complementation experiments is also shown.

supported by the fact that both organelles contain a mitochondrially related chaperonin CPN60, lack an organellar genome and have lost most enzymes associated with aerobic metabolism. Moreover, like some type II amitochondriate eukaryotes, *E. histolytica* is an aerotolerant anaerobe and metabolizes pyruvate via the enzyme pyruvate:ferredoxin oxidoreductase (PFO), a benchmark of anaerobic energy metabolism.

The persistence of a mitochondrially related organelle in *E. histolytica* indicates that this organism is not, as previously thought, a type I amitochondriate eukaryote. However, it is not a type II amitochondriate either, as pyruvate oxidation occurs in the cytoplasm (Takeuchi *et al.*, 1975; Reeves *et al.*, 1977) and energy preservation through electron transport has not been demonstrated (Reeves, 1984). Thus, it appears that *E. histolytica* is currently in a transitional state of evolution between a type II and a type I amitochondrial organism, implying that the mitosome may eventually be lost from *Entamoeba*.

The physiological functions of the mitosome are at present unknown, but it is unlikely to represent a separate compartment of energy metabolism like the mitochondrion or the hydrogenosome. In *E. histolytica*, glucose is converted to pyruvate via the classical Embden–Meyerhof pathway, and all participating enzymes are soluble and are not associated with subcellular compartments (Reeves, 1984). The oxidative decarboxylation of pyruvate also occurs in the cytoplasm, as supported by the purification of the *E. histolytica* PFO enzyme from soluble extracts (Takeuchi *et al.*, 1975; Reeves *et al.*, 1977). In contrast, *Trichomonas* PFO resides in the hydrogenosome (Williams *et al.*, 1987) and the *Giardia* enzyme appears to be associated with membranes (Ellis *et al.*, 1993; Townson *et al.*, 1996). A recent report identified an *Entamoeba* PFO-like protein associated with cellular membranes using a polyclonal antibody (Rodriguez *et al.*, 1998). However, catalytic activity was not demonstrated by these authors, nor has any significant proportion of activity been detected in membranous fractions by others (Takeuchi *et al.*, 1975; Reeves *et al.*, 1977).

Conservation of a mitochondrion-like protein import mechanism in the *E. histolytica* mitosome indicates that this organelle is not a peroxisome-like microbody, as peroxisome-targeted proteins contain a C-terminal signal sequence required for protein import into the organelle. Peroxisomes have not been detected in *Entamoeba* and are believed to be of evolutionary origin unrelated to mitochondria (Rachubinski and Subramani, 1995). In conclusion, we have identified a novel organelle in the amitochondrial parasite *E. histolytica* that shares a common evolutionary ancestor with mitochondria and hydrogenosomes. The name mitosome has been chosen to indicate that this microbody is related to mitochondria. The relationship, if any, between the mitosome and 'mitochondrial bodies' detected decades ago by vital staining and cytochemistry is unknown (Causey, 1925; Ray and Sen Gupta, 1954). Ultrastructural studies and a thorough biochemical characterization of pure mitosomal preparations are required to determine the fine structure and physiological function(s) of this novel organelle.

Experimental procedures

Parasite culture and transfection

Entamoeba histolytica HM-1:IMSS clone 9 was maintained axenically by subculture in YI-S medium with 15% adult bovine serum as described (Diamond *et al.*, 1995). Transfection of trophozoites by electroporation and selection of recombinant clones in G418-containing medium was carried out exactly as described (Hamann *et al.*, 1995).

RNA manipulations and analysis

Messenger RNA was isolated using a QuickPrep Micro mRNA purification kit (Pharmacia). For analysis, 0.5 μg aliquots of mRNA were size-fractionated in formaldehyde-containing 1% agarose gels (NorthernMax, Ambion). RNA size markers (Promega) were electrophoresed in an adjacent lane and subsequently stained with ethidium bromide. RNA was transferred to nylon membranes (BrightStar-Plus, Ambion) and hybridized by conventional methodology (Sambrook *et al.*, 1989) to high

specific activity radiolabelled probes (Feinberg and Vogelstein, 1984).

Cloning and sequencing of the E. histolytica cpn60 gene

A genomic clone (p5′gcpn60) containing a 2.1 kb partial sequence of the *E. histolytica* cpn60 gene locus was identified by screening a size-selected library (Descoteaux *et al*., 1992) with a radiolabelled cpn60 internal fragment previously described (Clark and Roger, 1995). The genomic clone contains 1.2 kb of the 5′ untranslated region and the first 875 nucleotides of the coding sequence and is marked by a unique *Eco*RI site at its 3′ end. The remainder of the coding region was obtained by reverse transcription-PCR amplification of a 0.8 kb fragment using a poly T anchor oligonucleotide and the internal primer CPN3 [5′-TGCAGTAAGAGCTCCAGG-3′]; this fragment was cloned into the pGEM-T vector system II (Promega) to generate plasmid p3′cpn60. The full cpn60 gene sequence was assembled by cloning the *Eco*RI–*Sal*I fragment from p3′cpn60 (containing the 3′ end of the gene) into the similarly digested plasmid p5′gcpn60. Both strands of the full-length clone were sequenced using an ABI Prism Automated DNA Sequencer (Perkin-Elmer) and internal oligonucleotides as primers.

Construction of expression plasmids

For protein expression in bacteria, plasmid pQEcpn60 was generated by directional cloning of the cpn60 coding region, amplified using primer set NTERCPN [5′-TATACAGCATGCTTTCATCTTCAAGTC-3′]/CTERCPN [5′-TATACAAGATCTATTAATTTCCTTTTTTATTGG-3′] into *Sph*I/*Bgl*II-digested expression vector pQE-70 (Qiagen). Restriction sites in primers are underlined. To add a carboxy-terminus c-*myc* epitope to recombinant protein, the cpn60 coding region was amplified from pQEcpn60 using primer set NTCPN60 [5′-TATAGGGATCCGCATGCTTTCATCTTCAAG-3′]/CTCPN + H6 [5′-TGCTCGATATCGTGATGGTGATGGTGATGAG-3′]; the amplified product was cloned into *Bam*HI/*Eco*RV-digested pTEX-9E10 (Tibbetts *et al*., 1995) to generate plasmid pB1. Plasmid pEhNEOplus (a kind gift from E. Tannich, Hamburg) was used as the basis for protein expression in *E. histolytica*. Construct A (Fig. 6A) was generated by subcloning the c-*myc*-tagged cpn60 sequence of pB1, amplified using primer set OT + [5′-TATAGGGTACCATGCTTTCATCTTCAAGT-3′]/3′BH1 [5′-TATAGGGATCCTTACAAGTCCTCTTCAGA-3′], into the *Kpn*I/*Bam*HI-digested pEhNEOplus. In an analogous fashion, Construct B (Fig. 6A) was generated by subcloning a truncated version of cpn60, amplified from pB1 with primer set OT-KPN [5′-TATAGGGTACCATGAATATTGATTGTAGA-3′]/3′ BH1 (see above), into pEhNEOplus as above. The addition of the HSP70 mitochondrial targeting signal to recombinant CPN60 was accomplished by amplification of a truncated version and a full-length copy of cpn60 from pQEcpn60 using primer sets OT-EV [5′-TATAGGATATCATGAATATTGATTGTAGA-3′]/CTCPN + H6 (see above) and NTCPN + MS [5′-TATTCGATATCCTTTCATCTTCAAGTC-3′]/CTCPN + H6, respectively, and subcloning into the *Eco*RV site of pTEX-9E10. The resulting plasmids pAF1 and pA7 were used as templates to amplify the chimeric cpn60 sequences using primer set MT + OT + [5′-TATAGGGTACCATGTTCGCTCGTCGTCGT-3′]/3′BH1. Constructs C and D (Fig. 6A) were derived by subcloning these amplified fragments from pAF1 and pA7, respectively, into pEhNEOplus as before.

Heterologous expression and protein purification

Poly His-tagged CPN60 recombinant protein was expressed in *Escherichia coli* (JM109) harbouring pQEcpn60 and partially purified under denaturing conditions by affinity chromatography using an Ni-NTA column (Qiagen). CPN60-enriched fractions were pooled and concentrated by microfiltration. Aliquots (0.5–0.6 mg) of partially purified recombinant protein were size-fractionated in 7.5% SDS–PAGE gels and electroblotted onto nitrocellulose in transfer buffer (10 mM CAPS, 10% methanol, pH 10.5). CPN60 was eluted from the membrane and concentrated to a final volume of 0.1 ml by microfiltration. Approximately 0.2 mg of protein purified to apparent homogeneity was recovered using this method.

*Preparation of anti-*E. histolytica *CPN60 serum and affinity purification of antibody*

Aliquots of 0.1 mg of purified recombinant CPN60 were used to generate polyclonal immune rabbit serum. Affinity purification of anti-CPN60 antibody was carried out by binding CPN60 onto nitrocellulose, incubating with a 1:20 dilution of polyclonal anti-CPN60 serum and eluting the antibody with 0.5 ml of 0.1 M glycine, pH 2.6, containing 1% bovine serum albumin. The antibody solution was immediately neutralized by the addition of 0.07 ml of 1 M Tris, pH 9.0, aliquoted and stored at −20°C until used.

Western blot analyses

Membrane-bound protein obtained by electrophoretic transfer or dot blotting was incubated with appropriate dilutions of affinity-purified anti-CPN60 antibody. A secondary anti-IgG antibody linked to alkaline phosphatase was used for detection. Colorimetric reactions were developed and visualized using BCiP and NBT.

Cell fractionation

A method for the resolution of rat liver organelles by differential centrifugation (Graham, 1993) was adapted for the fractionation of *E. histolytica* extracts. All purification steps were carried out at 4°C and in the presence of the protease inhibitors *p*-hydroxymercuribenzoic acid (p-HMB, 1 mM), trypsin inhibitor (0.4 mg ml^{-1}), leupeptine (0.75 μg ml^{-1}) E64 (70 μM) and E64c (8 μM). Exponentially growing cells were collected by chilling in an ice-water bath and centrifugation at 275 × *g* for 3 min, washed in PBS and resuspended in isotonic buffer (0.2 M mannitol, 50 mM sucrose, 10 mM KCl, 1 mM EDTA, 10 mM HEPES pH 7.4) at a density of 5×10^6 cells per millilitre. Cells were gently lysed on ice using 8–12 strokes in a 3 ml Dounce glass homogenizer, and the extracts were fractionated by differential centrifugation into a nuclear fraction (5 min at 1000 × *g*), a 'mitochondrial' fraction (10 min at 3500 × *g*), a

heavy microsomal fraction (10 min at 5000 × *g*) and a light microsomal fraction (10 min at 10 000 × *g*). The supernatant from the last centrifugation was precipitated with 5 vols of cold acetone, and the final pellet was resuspended in buffer to generate the cytosolic fraction. Centrifugation of fractions and precipitation of protein with acetone were repeated three times to minimize organellar cross-contamination.

Trypsin protection assay

Microsomal fractions were prepared as above except that no trypsin inhibitor was used. Microsomes were resuspended in 0.8 ml mannitol buffer and split into 4 × 0.2 ml aliquots. One aliquot was kept on ice as control, a second aliquot received trypsin (20 μg ml^{-1}) only, whereas a third and a fourth received trypsin plus Triton X-100 (0.2%). Before the addition of trypsin and detergent, aliquot 4 was supplemented with trypsin inhibitor (800 μg ml^{-1}). After 20 min of incubation on ice, trypsin inhibitor (800 μg ml^{-1}) was added to all samples. Organelles were collected by centrifugation at 14 000 r.p.m. for 10 min and resuspended in SDS–PAGE sample buffer. After electrophoretic separation, the presence of CPN60 was monitored by Western blotting.

Immunofluorescence microscopy

Trophozoites (10^4 cell aliquots) were placed on 3-aminopropyl triethoxysilane (APES)-coated slides and fixed in 4% paraformaldehyde for 45 min. Cells were permeabilized in 1% Triton X-100 for 30 min and blocked in 5% BSA for 60 min. Preparations were incubated overnight with an anti-c-*myc* monoclonal antibody (Santa Cruz Biotechnology) and, after repeated washing with PBS-Triton, with a fluorescein-conjugated anti-mouse antibody. Cells were finally incubated in a 1 mg ml^{-1} propidium iodide solution for 5 min, washed repeatedly in PBS and examined under a Leitz Diaplan fluorescence microscope.

Accession number

The nucleotide sequence data reported in this paper has been deposited in the DDBJ/EMBL/GenBank databases under accession number AF029366.

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Note added in proof

After this paper was accepted for publication a report on a similar *E. histolytica* organelle appeared in print (Mai, Z., Ghosh, S., Frisardi, M., Rosenthal, B., Rogers, R., and Samuelson, J. (1999) Hsp60 is targeted to a cryptic mitochondrion-derived organelle ('crypton') in the microaerophilic protozoan parasite *Entamoeba histolytica*. *Mol Cell Biol* **19:** 2198–2205).

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