Curr Genet (2003) 44: 138–147
DOI 10.1007/s00294-003-0432-1

RESEARCH ARTICLE

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Cryptosporidium parvum Cpn60 targets a relict organelle

Received: 26 March 2003 / Revised: 30 June 2003 / Accepted: 13 July 2003 / Published online: 20 August 2003
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Abstract Chaperonin 60 (Cpn60) is a well-established marker protein for eukaryotic mitochondria and plastids. In order to determine whether the small double-membrane-bounded organelle posterior to the nucleus in the apicomplexan *Cryptosporidium parvum* is a mitochondrion, the Cpn60 gene of *C. parvum* sporozoites (*CpCpn60*) was analyzed and antibodies were generated for localization of the peptide. Sequence and phylogenetic analyses indicated that *CpCpn60* is a mitochondrial isotype and that antibodies against it localize to the rough endoplasmic reticulum-enveloped remnant organelle of *C. parvum* sporozoites. These data show this organelle is of mitochondrial origin.

Keywords Apicomplexan · Relict mitochondrion · Chaperonin 60 (Cpn60)

Introduction

Cryptosporidium parvum belongs to the diverse group of intracellular apicomplexans that includes species of human (*Babesia microti*, *Plasmodium falciparum*, *Toxoplasma gondii*) and veterinary (*Eimeria tenella*, *Neospora caninum*) importance (Ellis et al. 1998; Fayer 1997). Phylogenetic analyses indicate that dinoflagellates and ciliates share a common ancestor with apicomplexans to form the group Alveolata (Cavalier-Smith 1983; Ellis et al. 1998; Sogin 1991). Within the Apicomplexa, the genus *Cryptosporidium* (together with the gregarines) is an early emerging branch (Barta 1989; Carreno et al. 1999; Zhu et al. 2000a), forming a monophyletic sister group to the clade comprising *P. falciparum* and *T. gondii* (Zhu et al. 2000a). While a typical single mitochondrion is present in most apicomplexans (McFadden 2003), its existence in *C. parvum* remained an open question (Aji et al. 1991; Tetley et al. 1998). Recently, however, we showed that sporozoites of *C. parvum* do contain a double-membrane-bounded organelle posterior to the nucleus that differs in its structure from typical mitochondria (Riordan et al. 1999). This organelle with an as yet unknown function is a possible candidate for a relict mitochondrion.

C. parvum appears to share with most apicomplexans extended glycolysis as the primary source of ATP, with little or no contribution by oxidative phosphorylation (Crawford et al. 2003; Entrala and Mascaro 1997; Martin et al. 2001). Unlike most eukaryotes, which contain many mitochondria, the apicomplexans *P. falciparum* and *T. gondii* contain a single mitochondrion (McFadden 2003) that retains a 6-kb organellar genome encoding only five proteins (Feagin 2000). As in all organisms, most mitochondrial proteins of the Apicomplexa are encoded by the nucleus, translated in the cytoplasm, and post-translationally imported into this mitochondrion (McFadden 2003). Both the mitochondrion of *P. falciparum* and the double-membrane-bounded organelle of *C. parvum* appear to generate a proton gradient, as indicated by the uptake of mitochondrion-specific vital fluorescent dyes (Crawford et al. 2003; Keithly, Ault, Buttle, Mannella, LaGier, Langreth, unpublished data). Unlike *P. falciparum* and *T. gondii* (Crawford et al. 2003), there is no evidence for an electron transport chain in *C. parvum*, but uptake of 10-N-nonyl acridine orange indicates that the *C. parvum*

Communicated by M. Brunner

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organelle contains cardiolipin, an important component of the mitochondrial inner membranes (IM).

Like eukaryotes, most apicomplexan mitochondria contain cristae with connections to the mitochondrial IM. The type and number of these cristae are differentially expressed during apicomplexan life cycles, ranging from tubular in *T. gondii* tachyzoites and *P. falciparum* gametocytes to acristate in asexual *P. falciparum* and *P. knowlesi* (Fry and Beesely 1991; Krungkrai et al. 1999, 2000). Electron tomography suggests the presence of cristae-like compartments in the *C. parvum* organelle (Keithly, Ault, Buttle, Mannella, LaGier, Langreth, unpublished data) but, unlike in its nearest relatives, these appeared to be unattached to the IM. Since the number of cristae tubular connections to the IM is predicted to correlate with global ATP production (Mannella et al. 2001), perhaps the distinctive cristae of the *C. parvum* organelle signals not only the loss of oxidative phosphorylation observed in all apicomplexan mitochondria, but also a significant divergence in organelle structure and function within the phylum.

Molecular evidence also suggests that this organelle may share a mitochondrial-type ancestry with other protists. First, the nucleus-encoded genes adenylate kinase 2 and valyl tRNA synthase [with affinities to mitochondrial homologues (Bui et al. 1996; Hashimoto et al. 1998; Sanchez and Muller 1998)], have been isolated and characterized (Riordan et al. 1999). Second, genes associated with the iron sulfur cluster (Isc) assembly [a known function of eukaryotic mitochondria (Lill and Kispal 2000)] have been isolated; and at least two of these (IscS, IscU) contain transit peptides that target green fluorescent protein (GFP) to yeast mitochondria (LaGier, Tachezy, Stejskal, Kutisova, Keithly, unpublished data).

Heat-shock protein or chaperonin 60 (Hsp/Cpn60) is the quintessential nucleus-encoded gene, indicating the endosymbiotic origin of eukaryotic mitochondria (Archibald et al. 2002; Gupta 1995; Horner and Embley 2001) from an α-proteobacterium (Bui et al. 1996; Martin et al. 2001). Previously, for example, the most convincing evidence for a mitochondrial origin of the "amitochondriate" *Trichomonas vaginalis* hydrogenosome (Bui et al. 1996) and the *Entamoeba histolytica* mitosome/crypton (Clark and Roger 1995; Mai et al. 1999; Tovar et al. 1999) was the presence of Cpn60 in their genomes and the subsequent localization of the chaperonin to these organelles.

Among the Apicomplexa, Cpn60 is encoded by the genomes of *Toxoplasma gondii*, *P. falciparum*, and *P. yoelii* (Sanchez et al. 1999; Syin and Goldman 1996; Toursel et al. 2000); and Cpn60 peptides have been localized by immunoelectron microscopy to the single mitochondrion of *T. gondii* tachyzoites (Toursel et al. 2000) and *P. falciparum* trophozoites within infected erythrocytes (Das et al. 1997). Here, we show that, like its nearest relatives, Cpn60 is encoded in the genome of *C. parvum* (*CpCpn60*) and, by sequence analysis, phylogenetic reconstruction, and localization of the CpCpn60 peptide, we show that the organelle of unknown function in *C. parvum* sporozoites is of mitochondrial origin.

Materials and methods

Organisms

Dr. M.V. Nesterenko (Kansas State University, Manhattan, Kan.) and P. Mason (Pleasant Hill Farm, Troy, Idaho) provided oocysts of *C. parvum* strains KSU-1 and Iowa, respectively. Oocysts were purified by CsCl or sucrose-gradient centrifugation, followed by 5 min sterilization in 10% Clorox on ice and 5–8 washes by centrifugation in sterile water. For in vitro excystation, purified oocysts were incubated at 37 °C for 1 h in Hanks′ balanced salt solution (HBSS) containing 0.25% trypsin and 0.75% taurodeoxycholate to obtain free sporozoites, as described by Keithly et al. 1997).

Total DNA or RNA was isolated from intracellular stages of *C. parvum* as detailed by Zhu et al (2000b). Briefly, to isolate total RNA from intracellular stages, human ileocecal epithelial cells (HCT-8; ATCC CCL 244) cultured for 18 h at 37 °C were inoculated with $3.0{\times}10^4$ sterilized oocysts, so that excystation/invasion could occur. Uninfected, control HCT-8 cells were treated identically.

Cloning, gDNA isolation, libraries, and sequencing

Genomic DNA (gDNA) was isolated from free sporozoites using DNAzol genomic DNA isolation reagent (Molecular Research Center). Both pBluescript SK(+) *Hin*dIII and *Eco*RI gDNA libraries of *C. parvum* KSU-1 strain provided by Dr. N. Khramtsov (Kansas State University) were screened for mitochondrion-specific genes. A set of degenerate oligonucleotide primers corresponding to highly conserved regions of the *CpCpn60* gene [sense: SK(I/V) TKGGCTV; antisense: L/P(A/P/S/T)Q(A/P/S/T)(G/R)(C/G/S/K) (Clark and Roger 1995)] were used in PCR with *C. parvum* gDNA to amplify a portion of the gene. The 576-bp amplicon was purified using a GeneClean Kit (Bio101) and then cloned into pCR2.1 (TA cloning kit; Invitrogen). The excised 576-bp insert was radiolabeled with [α-^{32}P]-dATP using the Random prime DNA labeling kit (Boehringer Mannheim) before probing gDNA libraries and Southern blots. Most of the sequence was obtained from several clones by gene-walking. The 5′ end of the gene was obtained using the GeneRacer kit (Invitrogen), in which complementary DNA (cDNA) ends are rapidly amplified with an interior primer designed from the known Cpn60 sequence. Sequences of each of the isolated clones were determined twice for both strands by an automated DNA sequencer within the Wadsworth Molecular Genetics Core Facility.

Southern blots and RT-PCR

For Southern blot analysis, 1 μg sporozoite gDNA/lane was digested with *Eco*RI, *Hin*dIII, *Eco*RI/*Hpa*II, or *Eco*RV, separated by electrophoresis and transferred to Zeta Probe Nylon membranes (Bio-Rad). A 576-bp DNA fragment of the *CpCpn60* ORF was released from a plasmid clone by restriction digestion with *Eco*RI, labeled with [α-^{32}P]-dATP, and used to probe blots under conditions of high stringency (Maniatis et al. 1982). RT-PCR used a pair of primers specific for *CpCpn60*, cpnRTfor (5′-ATC-ACC-GAA CCC-TGG-CGC-TTT-TAC-TGC-3′) and cpnRTrev (5′-GTG TTA-GCA-AGA-GCA-ATT-TTC-AAA-TCA-3′), which produced an amplicon of 576 bp. Each 50-μl reaction included 5 units of AMV reverse transcriptase, 5 units of *Tfl* DNA polymerase, 100 ng of total RNA isolated from *C. parvum* sporozoites using the RNeasy kit (Qiagen), 1 μM of each primer, and other reagents as suggested for the Access RT-PCR kit (Promega). Two negative controls containing all reagents except total RNA or reverse transcriptase, respectively, were always included. The first-strand

cDNA synthesis was conducted at 48 °C for 45 min, followed by heat-inactivation of the reverse transcriptase at 94 °C for 2 min. The cDNA was then amplified for 35-cycles. All amplicons were sequenced to confirm identity.

Sequence alignments and phylogenetic analysis

Preliminary alignments were performed using the GCG Wisconsin package ver. 9.1 within the UNIX cluster at the Wadsworth Center. The alignment was improved by visual editing; and phylogenetic analyses were performed using the program packages MUST ver. 1.0 (Philippe 1993) and MOLPHY ver. 2.3 (Adachi and Hasegawa 1996). Alignments are available upon request.

Phylogenetic relationships among related sequences of *CpCpn60* were inferred by the maximum likelihood (ML) method of protein phylogeny (Kishino and Hasegawa 1989). The analysis was performed with the PROTML ver. 2.3 program; and the Jones–Taylor–Thornton model (Jones et al. 1992) was assumed for substitutions. Because the number of taxonomic units analyzed was very large, a neighbor-joining (NJ) tree (Saitou and Nei 1987) was first constructed, using a distance matrix estimated by the ML method. The NJ tree was then further analyzed by ML local rearrangements.

Yeast GFP plasmid

The mitochondrion-targeted plasmid pYX223-mtGFP (Westermann and Neupert 2000) was a gift from B. Westermann (Ludwig-Maxilians-Universitat Munchen, Munich, Germany). This plasmid contains a known mitochondrial pre-sequence from protein 9 (proteolipid subunit) of the F0 part of the F1F0 ATPase of *N. crassa* ligated to GFP (pSu9-69-GFP) and has an inducible GAL promoter which can be regulated. The first 170 bp (57 aa) of the *CpCpn60* gene were generated by PCR from gDNA by adding *Eco*RI and *Bam*HI linker sequences to the 5′ and 3′ end, respectively. The *N. crassa* mitochondrial pre-sequence was digested from the plasmid using *Eco*RI and *Bam*HI and was then replaced with the putative *C. parvum* pre-sequence. The new recombinant plasmid (pCpn60-57-GFP) was amplified in Ultracompetent XL-10 gold cells (Stratagene), purified, and sequenced to confirm its identity. It was then transfected into *Saccharomyces cerevisiae* (strain KY527) and plated onto galactose-containing *S. cerevisiae*-His agar to induce expression of the GFP. Only cells transformed with the recombinant plasmid grew on selective plates; and samples from these colonies were prepared for examination by attaching cells to glass slides coated with poly-L-lysine. Cells were observed with epifluorescence and phase contrast microscopy, using an Olympus BH2 phase/fluorescence microscope.

MitoTracker red vital fluorescent dye staining

MitoTracker red CMSRos (M7512) was used, as per the manufacturer′s instructions (Molecular Probes), to delineate the mitochondrial tubular network of the yeast *S. cerevisiae* transformed either with the recombinant plasmid pCpn60-57-GFP or with pSu9-69-GFP. This dye is well suited for multicolor-labeling experiments, because the red fluorescence ($\lambda_{ex} = 579$ nm, $\lambda_{em} = 599$ nm) is clearly resolved from the green fluorescence of other probes. Furthermore, the reduced probes fluoresce only upon entry into an actively respiring cell, where they are oxidized to the fluorescent mitochondrion-selective probe, which is then sequestered within the mitochondrial network.

Antibodies

The Wadsworth Center Peptide Synthesis Core Facility synthesized peptides corresponding to amino acids (aa) 67–84 and 230–248 of the *C. parvum* Cpn60 sequence. The peptides were selected using antigenicity indices, hydrophilicity, surface probability, and secondary structure criteria. These values were determined using the programs PEPTIDESTRUCTURE and PLOTSTRUCTURE in the GCG Wisconsin package ver. 9.1 within the UNIX cluster at the Wadsworth Center, together with BLASTP and CN3D ver. 2.5 (a molecular modeling database) at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/blast). These programs indicated that the selected peptides had little homology to other proteins and were specific for *C. parvum*. The selected regions of the protein also lacked significant secondary structure, were surface-oriented, and were therefore probably available for antibody-binding. The peptides were conjugated to Mariculture keyhole limpet hemocyanin (mcKLH; Pierce) and sent to Lampire Biological Laboratories (Pipersville, Pa.), where they were injected into two female New Zealand white rabbits (2 kg each, 12 weeks old) to produce antibodies. Each 1.0-ml primary and booster injection contained approximately 200 µg of conjugated peptide (0.5 ml of conjugate solution) and 200 µg of adjuvant (0.5 ml). Injections were distributed over four sites (250 µl/site). After the initial injection, rabbits were boosted once per month for 3 months and the rabbit serum was tested for reactivity to CpCpn60 and antibody titer by ELISA. Antisera of both rabbits showed a significant signal above background; and the pooled antiserum was affinity column-purified, using the peptide conjugated to agarose beads according to Lampire protocols (http://www.lampire.com).

The antibodies were first tested for specificity by Western blot analysis, using whole-protein extracts of *C. parvum* sporozoites. Later, sporozoites fixed in 10% formalin were washed with PBS and then adhered to glass slides coated with poly-L-lysine . Fixed slides were washed with methanol, blocked with 2% bovine serum albumin (BSA), and incubated with varying dilutions of rabbit pre-immune serum or affinity-purified antibodies diluted in 1% BSA/PBS and incubated overnight at 4 °C. Rinsed slides were then incubated with goat anti-rabbit IgG secondary antibody conjugated to fluorescein isothiocyanate (FITC) for 1 h at room temperature. Fluorescence was observed using an Olympus BH2 phase/fluorescence microscope.

Western blots

C. parvum sporozoites were excysted from oocysts and purified as detailed by Keithly et al. (1997). An aliquot of 2×10^7 sporozoites was lysed in standard SDS-reducing buffer at 100 °C for 10 min. Samples were centrifuged at 20,000 *g* for 1 min and the supernatant was loaded onto a 10% SDS-polyacrylamide gel along with appropriate ladders. The samples were run at 100 V/cm in the stacking gel and 200 V/cm in the separating gel. Separated proteins were transferred to a nitrocellulose membrane (Zetaprobe). Blots were probed with either rabbit pre-immune serum or purified antibodies at dilutions of 1:30,000 and were visualized using the Western blot chemiluminescence reagent plus kit (NEN Life Science Products) and Kodak X-Omat blue autoradiography film.

Immunoelectron microscopy

Sporozoites were fixed immediately after excystation in 4% electron microscopy (EM)-grade methanol-free formaldehyde (Polysciences) in HBSS at 4 °C for 16 h, followed by additional fixation in 4% formaldehyde/0.1% EM-grade glutaraldehyde (Polysciences) in 0.1 M Na cacodylate buffer containing 4% sucrose and 0.05 mM $CaCl_2$ (pH 7.4) at 4 °C for 3 h. Sporozoites were given four washes in this buffer (15 min each), dehydrated in an ethanol series, and embedded in LR White resin. Semi-thin (0.08–0.20 µm) sections were cut using a Diatome diamond knife on a Reichert Ultracut E ultramicrotome. The sections were blocked for 30 min in a solution containing 50 µg reconstituted whole goat serum/ml and 20 µg BSA/ml in Tris-buffered saline (TBS; containing 20 mM

Tris, 0.1% BSA, 0.05% Tween 20, 150 mM NaCl, 20 mM NaN_3, pH 7.4), exposed overnight at 4 °C to a 1:50 dilution Cpn60 affinity-purified rabbit antibody (58 μg/ml) in TBS (pH 7.4), washed four times in TBS and labeled for 1 h with a 1:100 dilution of EM-grade 10-nm gold particles conjugated to goat anti-rabbit IgG antibodies (British Biocell International, via Ted Pella) in TBS containing 10 μg reconstituted whole goat serum/ml. Sections were then washed five times in TBS, exposed to 1% gluteraldehyde in water for 5 min (to covalently link the primary antibody to the secondary one), stained with 2% aqueous uranyl acetate for 20 min, and finally washed three times in water. Preincubation with a 1:50 dilution of CpCpn60-specific antibody (58 μg/ml) plus 100 μg CpCpn60 peptide/ml to block specific labeling served as the negative control. All other treatment steps were identical. Substitution of a rat polyvalent antiserum raised against purified *C. parvum* sporozoite membranes as the primary antibody, followed by treatment with 10-nm gold particles conjugated to goat anti-rat IgG, served as a positive control. Sections were examined at 80 kV with a Zeiss 910 transmission electron microscope.

Statistical analysis

Samples used for analysis were transmission electron micrographs of individual, longitudinal sections of whole sporozoites, including both the posterior and anterior ends. Calculations were based upon the number of gold particles bound per square micron over the area of the putative relict mitochondrion versus gold particles over the remaining area of the sporozoite. Thirty-four positive sporozoites shown in longitudinal section (treated with CpCpn60-specific antibody alone) and 24 negative sporozoites (treated with CpCpn60-specific antibody plus blocking peptide) were used for statistical analysis. The number of gold particles bound per square micron were analyzed by Student's *t*-test to determine statistical significance.

Results

Characterization of the *CpCpn60* gene

The 1,857-bp *CpCpn60* gene is 63% AT-rich, which is characteristic of *C. parvum* genes, and encodes a protein of 619 aa. The protein, predicted to have a mass of 66 kDa, has a high overall identity to other Cpn60 sequences and includes aa residues that are specifically conserved among mitochondrion-derived sequences. An alignment of representative chaperonins (Fig. 1) includes sequences from the "mitosome"-bearing entamebid *E. histolytica*, the "amitochondriate" diplomonad *Giardia intestinalis*, the mitochondrion-bearing apicomplexan *P. falciparum*, the chloroplast- and mitochondrion-bearing red alga *Porphyra purpurea*, the mitochondrion-bearing *Homo sapiens*, and the eubacterial GroEL sequence from *Escherichia coli*. The Mg^{2+}-ATP binding regions contain the most conserved aa and are boxed in Fig. 1. Furthermore, there are 12 aa residues known to be essential for substrate-binding in the *E. coli* GroEL (Braig et al. 1994; Fenton et al. 1994) sequence (Fig. 1, asterisks); and there are an additional 10 aa thought to be contact points between GroEL (Cpn60) and the co-chaperone GroES (Cpn10) (Braig et al. 1994; Brocchieri and Karlin 2000) that are also found in the *C. parvum* sequence (Fig. 1, circumflexes). Somewhat unexpectedly, eight of these ten are more highly conserved between CpCpn60 and other eukaryotic (*H. sapiens*, *P. porphyra*) or bacterial (*E. coli*) mitochondrial sequences than they are to those of other protists (*Entamoeba histolytica*, *G. lamblia*, *Plasmodium falciparum*). Analyses of the *Escherichia coli* GroEL crystal structure (Brocchieri and Karlin 2000) predicts that 48 aa exposed to the inside barrel of the double-ring complex should be highly charged; and indeed 35 of them show charge conservation (Fig. 1, hashmarks). The *C. parvum* sequence shows overall similarity to mitochondrial sequences (Horner and Embley 2001). There is a 20-aa serine/proline (SP)-rich insert beginning at aa 472 in CpCpn60 not found in most other sequences. Interestingly, the more divergent of two Cpn60s found in the apicomplexan *P. falciparum* also contains an insert, but it is not SP-rich. The functional significance of these inserts in the Apicomplexa is not known.

Unlike the apicomplexans *T. gondii* (Toursel et al. 2000), *P. yoelii* (Sanchez et al. 1999), and one Cpn60 *P. falciparum* isoform (Syin and Goldman 1996), *C. parvum* lacks a C-terminal glycine/methionine (GGM) repeated motif commonly found in a broad spectrum of species (Hemmingsen et al.1988; McLennan et al. 1993). Instead, *C. parvum* and the second Cpn60 *P. falciparum* isoform encode either a serine-rich or glutamine/aspartate-rich C-terminal tail (Fig. 1, overlines), while most other protists and plastid-bearing eukaryotes completely lack any extended motif (Fig. 1).

Southern blot and RT-PCR analysis of gene expression

Southern blot analysis using gDNA extracted from *C. parvum* sporozoites digested with several restriction enzymes and probed with the radiolabeled PCR fragment of the *CpCpn60* gene described previously show a single band (Fig. 2A), suggesting that *CpCpn60* is a single-copy gene. RT-PCR with RNA from both free sporozoites and intracellular parasites showed that CpCpn60 RNA is transcribed (Fig. 2B). The absence of a 576-bp product in negative controls lacking either reverse transcriptase or RNA, including uninfected HCT-8 cells, confirms that transcription is parasite-mediated. Although the 576-bp band amplified from infected HCT-8 cells is somewhat brighter than that from sporozoites, it does not indicate differential expression of the protein, because the amounts of RNA were not adjusted to identical concentrations in these reactions.

Phylogenetic affinities

The phylogenetic relationship among major Cpn60 clusters was examined using a dataset of 47 sequences that included Cpn60 from *C. parvum* and three other apicomplexans, six from other protists (two *T. vaginalis* isoforms), five from α-proteobacteria, six from γ-proteobacteria, and 25 from other taxa (Fig. 3). The topology

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                                          ->                                   #                    <-
Cp ---------- ---------- ---------- ---MLLRSGI NLYKSVEGSI GLRSAAIRFG MR--YISSGK ELSFGGKARK EMLKGANDLA  55
Eh ---------- ---------- ---------- ---------- ---------- ---------M LSSSSHYNGK LLSLNIDCRE NVLSGIKKVA  31
Gi ---------- ---------- ---------- ---------- ---------- ---------- --MLQHYTSV ISGE--DARS GLLRGIKTIA  26
Pf MRMKRIHILF VVIFLLCLRY GYSIKKKRSP NNKNRLFINK RLKYINSKII SRRKENYVKM KMTENKVKGK DIIYGNECRN ELLKGILTVS  90
Pp ---------- ---------- ---------- ---------- ---------- ---------- -------MSK QILYQDDARK ALEKGMDILT  23
Hs ---------- ---------- ---------- ----MLRLPT VFRQMRP--- -VSRV-LAPH LTRAYA---K DVKFGADARA LMLQGVDLLA  48
Ec ---------- ---------- ---------- ---------- ---------- ---------- ------MAAK DVKFGNDARV KMLRGVNVLA  24

                     #                                                         #
Cp DAVGVTLGPR GRNVVI-EQG FGEAPKITKD GVTVAKAIQF GKKGSVNLGAQ LLKNVAISTN EEAGDGTTTA TVLARAIFKS GCEKVDAGLN 144
Eh DAVSVTLGPK GRTVII-DQP YGNA-RVTKD GVSVAKALTF SDNTLNVGGK IAKEVASKVN DRSGDGTTTA TCLLRKVACE GVQAINTGLS 119
Gi DVVATTLGPR GRAVILADGS ASGTTKVTKD GVSVARAINL S-GLEGVGAD LIKDASLRTN TMAGDGTTTS LILSGKLVNE MNKYALSGLG 115
Pf DVVKLTLGPR GRNVLL-EKE YG-SPLIIND GVTIAKNISL KDRKKNNGVK LMQESTNISN DKAGDGTSST ALMTATITKK GIEQVNRNHN 178
Pp EAVSVTLGPK GRNVVL-EKK FG-APQIVND GVTIAKEISL EDHIENTGVA LIRQAASKTN DVAGDGTTTA TVLASAIVKQ GMRNVAAGSN 111
Hs DAVAVTMGPK GRTVII-EQS WG-SPKVTKD GVTVAKSIDL KDKYKNIGAK LVQDVANNTN EEAGDGTTTA TVLARSIAKE GFEKISKGAN 136
Ec DAVKVTLGPK GRNVVL-DKS FG-APTITKD GVSVAREIEL EDKFENMGAQ MVKEVASKAN DAAGDGTTTA TVLAQAIITE GLKAVAAGMN 112

                                                                               #         #      ## * *
Cp PMDLLRGIKL GVEHVVNELD LLSQPVKSHD DILNVATISA NGDSIVGSLI AQAYSKVGRH GTINIEEGNT TQSELEIVEG LKLDKGYISP 234
Eh GTDLLKGISI AKDIVLKEIT KQSKP-TLKE DIISVARVSA NNDEKIGEMV GDIFGKIGRD GAVDIETGKG TKDIVNIVEG MVLDQGFLSR 208
Gi NLQLLQALNS AGVDCLQSLR KQSRAIESNK MLYSVATIAA NNDPKIGKVV SDAFAAVGRE GTITVEDGYT DIDTLNVTDG CSIPSGFLSP 205
Pf PIPIQRGIQL ASKMIIEKIK SLSTPIKTYK DILNIATIAS NNDVHMGQII ANAYDKLGKN AAIILDDNAD INDKLEFTEG YNFDRGIINP 268
Pp PMAIKKGIEK ATNFVVSKIA EYAKPVEDTT AIVQVASISS GNDAEVGKMI ADAIDRVGRE GVISLEEGKS TSTSLEITEG MQFEKGFISP 201
Hs PVEIRRGVML AVDAVIAELK KQSKPVTTPE EIAQVATISA NGDKEIGNII SDAMKKVGRK GVITVKDGKT LNDELEIIEG MKFDRGYISP 226
Ec PMDLKRGIDK AVTAAVEELK ALSVPCSDSK AIAQVGTISA NSDETVGKLI AEAMDKVGKE GVITVEDGTG LQDELDVVEG MQFDRGYLSP 202

   **                 ###         *  **                ## # # *    *  *  *  *      #      # ##
Cp YFITNQKYQK VELENPYILI SQGKISSL-K SILPILEFCI SSRSPLLIIA EEIEGEALTA LILNKLQLNL KVCAVKAPGF GDHRKQILED 323
Eh YFTTDEKNTK VDIRNTDVIV CDYKLSS-SQ SVVPLLELCL KRKRPLVVIS DTIDGDALTT LVLNKLRG-L PIAAVRAPGF GETRKGILHD 296
Gi YFALGG-SRY LELTNPLVVI TDTVLSSAA- PLVSILERCV KEKRPLLIIA SDVTGDALST LAINTLKGTV RCCAVRAPGY GDVKKGVLED 293
Pf YLLYNENKDY IEYSNVSTLI TDQNIDNI-Q SILPILEIFA KNKQPLCIIA DDFSNEVLQT LIINKLKGAI KVLCIVT--- --NSKYISAD 352
Pp YFVTDLDRME VLQENPFILF TDKKITLVQQ ELVPLLEQIA KTSKPLLIIA EDIEKEALAT IVVNKLRGIL NVVAVRAPGF GDRRKSLLED 291
Hs YFINTSKGQK CEFQDAYVLL SEKKISSI-Q SIVPALEIAN AHRKPLVIIA EDVDGEALST LVLNRLKVGL QVVAVKAPGF GDNRKNQLKD 315
Ec YFINKPETGA VELESPFILL ADKKISNI-R EMLPVLEAVA KAGKPLLIIA EDVEGEALAT LVVNTMRGIV KVAAVKAPGF GDRRKAMLQD 291
                                  ^  ^^  ^ ^                   ^   ^    ^

                ##                             ##                                   # #  ##  ##  #
Cp ISVSVG-AKI IQEEFSNAKL DQMNSNQIQE FLGKCKSISV SKDETII--T QGQGSPKDVK DTISLLKSQI EENQKLTDYD KEKLRERLAR 410
Eh IGIITG-ATV ISNE-AGKKI EEVT----EK DLGKIGHFVS TKDETII--T GGAGSKAEVL ARINELKNAK EV--SDSSYE KEKLEGRIAR 376
Gi LAAVVGIPTY ISDE---LHT ASAPGSAVLS NIGSCHKAII TPANTVLHFN DDKNCNSLIR GRVAGLRSLL E-SNNLTNYQ RSKLNERIGR 379
Pf VGLDLN---N LHNNMSSFDN NYL------S LLGSANTLIV KKDRTSL--I TKEEYKKEID ERINVLKKEY EETT--SKYD KEKLNERIAA 429
Pp MSILTG-GQV ITED-AGFSL DTV---QL-D MLGKARRVVV TKDSTTI--I -ADGHEATVK SRCEQIKRQI E-TSD-SLYE REKLQERLAK 370
Hs MAIATG-GAV FGEEGLTLNL EDV---QPHD -LGKVGEVIV TKDDAML--L KGKGDKAQIE KRIQEIIEQL DV-TT-SEYE KEKLNERLAK 396
Ec IATLTG-GTV ISEE-IGME LEKA---TL-E DLGQAKRVVI NKDTTTI--I DGVGEEAAIQ GRVAQIRQQI E-EAT-SDYD REKLQERVAK 371
                      ^        ^                               ^          ^

            #      #       #          #   #                                    _________________
Cp LTGRVALIKI GGYSDTEISE LKDRFIDALN ATKCAIEQGI VPGGGSALLW ASRN-----L GKLYSQSPPP GKTLTPSQSS SNESNPIR-N 494
Eh LTGGVAVISV GGSSEAEVGE RKDRIEDAVC AVKAALAEGI VPGGGVALIR AGSS-----L DKIR------ ---------- --SQNWA--- 440
Gi LLGKVCTIRI GAKTELEAEE KKDRYIDSLS AARAALEGGL LPGGGVAFLR AAQV-----M ERKL------ ---------- --AEGKVA-- 444
Pf LSGGIAKILI GGNSETEQKE RKFKYEDATN AVKSAIDIGY VPGGGVTYLE IIKSNFIQEI HKKIEEDLQI SSNNDEKKYL ELIGNLESEM 519
Pp LSGGVAVIKV GAATETEMKD KKLRLEDAIN ATKAAIEEGI VPGGGSTNVH ISGE-----L FVW------- ---------- --AKN-NL-F 434
Hs LSDGVAVLKV GGTSDVEVNE KKDRVTDALN ATRAAVEEGI VLGGGCALLR CIPA-----L DSL------- ---------- --T---PA-N 458
Ec LAGGVAVIKV GAATEVEMKE KKARVEDALH ATRAAVEEGV VAGGGVALIR VASK-----L ADL------- ---------- --RGQ-N--- 433

Cp YDMAMGVKIV QDACK---VP CHLISSNAGF DGSVIVGELV KVFSKGSKHF GFDAQTGQFV DMIESGILDP TKVVKSGLRD AASIASLMTT 581
Eh --EKVGIDIV RKVTE---EP TRIIARNAGI DGGIVIQKIK EGTGS----F GYDVRKNVYC DLMKVGIVDP TKVVRNAFNE AISVGSLIAT 521
Gi --DPVTIAAH KALIAALHEP ARIIAESAGA SGHVVAEAI- --KNSPDNFY GFDALNGQFV NMEKAGILDA TKVVTTALDS ALGVSSVLLN 529
Pf ELQKMGANIV VSSLD---VI TKQIADNAGV NGDNVVKIIL N--SKDKYGF GYDVNTNKFV NMVEKGIIDS TNVIISVIKN SCSIASMVLT 604
Pp EDELIGALIV QRALT---YP LRRIAFNAGD NGAVVVEKV- --KTNDFC-I GYDASNGKIV NMYDAGIIDP AKVARSALQN ATSIAAMVLT 517
Hs EDQKIGIEII KRTLK---IP AMTIAKNAGV EGSLIVEKIM QSSSE----V GYDAMAGDFV NMVEKGIIDP TKVVRTALLD AAGVASLLTT 541
Ec EDQNVGIKVA LRAME---AP LRQIVLNCGE EPSVVANTV- --KGGDGN-Y GYNAATEEYG NMIDMGILDP TKVTRSALQY AASVAGLMIT 516
                 ____________________________________________________________________________

Cp TQVSVFE-PS NQSEKNNSSG SNSSESSSSF GSLPGDFYX- ---------- ---------- ---------- ---------- ---------- 619
Eh SEALITDEPI KKEIN----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 536
Gi TDAVVQPIPT DTNLFKNK-- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 547
Pf TECMMVDHEK KDKGILDSSI NSPNYLSKHR RTYKHKLHDD EDTDEDDEED EDDEDDEDDL DDDDYDDEDE EDEEDEEDED DEDDEDSMND 694
Pp TECIVVD--K LE-A------ ---------- ---------- ---------- ---------- ---------- ---------- ---------- 528
Hs AEVVVTEIPK EEKDPGMGAM GGMGGGMGG- -GMF------ ---------- ---------- ---------- ---------- ---------- 573
Ec TECMVTDLPK ND-AADLGAA GGM-GGMGGM GGMM------ ---------- ---------- ---------- ---------- ---------- 548
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of this ML tree essentially agrees with that for the *C. parvum* mitochondrial gene *AK2* (Riordan et al. 1999) and places all Cpn60 homologues into four major clades (Fig. 3). As in previous analyses, α-proteobacteria were recovered as a sister group to the mitochondrion-bearing eukaryote clades, consisting of protists, animals, fungi, and plants (Fig. 3) with strong support [bootstrap proportions (BP)≥85%], suggesting a common ancestral origin. Both γ-proteobacteria and plastid/chloroplast sequences are monophyletic to and distinct from them. Within the mitochondrial clade, the close phylogenetic relationship of animals and fungi is also confirmed (BP≥85%), but relationships among protists are less clearly resolved.

The two most likely reasons for the poor resolution among protists in this study are: (1) lack of species diversity in the Cpn60 database and (2) presence of lineages which are often fast-evolving and which can be misplaced due to long-branch attraction (LBA) artifacts (Philippe and Laurent 1998). LBA artifacts could be responsible for relationships here among entamebids, trichomonads, and diplomonads (Fig. 3). There is a weakly supported relationship between the Cpn60 of *T. vaginalis* and *G. lamblia* (BP = 40%), suggesting a potential sister relationship between them that has been found in other analyses (Hashimoto et al. 1998; Roger et al. 1998; Horner and Embley 2001). The placement of *C. parvum* as a sister group to the apicomplexans *Toxo-*

Fig. 1 Amino acid alignment of Cpn60 sequences from the protists *Cryptosporidium parvum* (*Cp*, GenBank AAC32614), *Entamoeba histolytica* (*Eh*, AAC38819), *Giardia intestinalis* (*Gi*, AAC38821), and *Plasmodium falciparum* (*Pf*, P34940), the red alga *Porphyra purpurea* (*Pp*, P51349), the mammal *Homo sapiens* (*Hs*, NP_002147), and the eubacterium *Escherichia coli* (*Ec*, AAC77103). Gaps in the sequences are *indicated by dashes*, *boxes* surround the regions implicated in Mg^{2+}-ATP binding, *asterisks* indicate residues conserved in most mitochondrial isoforms and for substrate-binding in *E. coli* GroEL, *hashmarks above the sequence* indicate residues exposed to the central cavity of GroEL and the conserved charge in most mitochondrial isoforms, *circumflexes below the sequence* indicate residues predicted to be involved in GroEL/GroES interactions. The mitochondrial pre-sequence of *C. parvum* is *shown in bold* and the N-terminal pre-sequence used for targeting GFP to yeast mitochondria is *shown by horizontal arrows above the sequence* at aa 1 (→) through aa 57 (←). An analysis of the alignment using the BLOSUM62 similarity matrix shows that *C. parvum* Cpn60 shares overall percent identity/similarity to chaperonin 60s of these organisms as follows: *H. sapiens* (43/63), *Esc. coli* (40/58), *Por. purpurea* (38/55), *Ent. histolytica* (36/58), *G. intestinalis* (29/49), and *Pla. falciparum* (28/44)

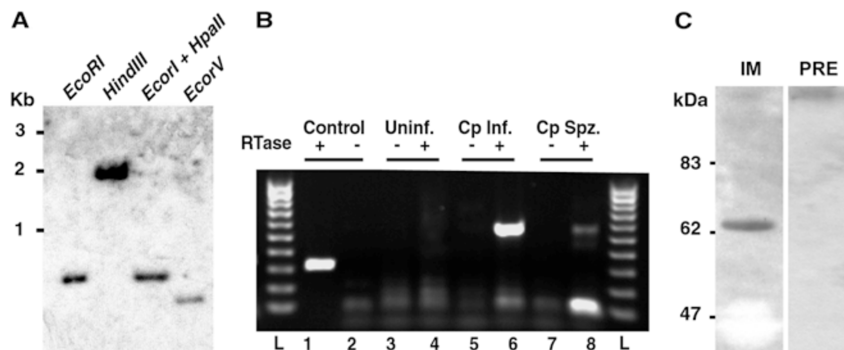
plasma and *Plasmodium* (BP≥85%; Fig. 3) is essentially congruent with other studies using six proteins, small subunit (SSU) rRNA, and large (L)SU/SSU rRNA, which show a trend for the early emergence of *Cryptosporidium* at the base of the Apicomplexa (Zhu et al. 2000a), with variations for sister group placements depending upon the protein analyzed.

Pre-sequence targeting and antibody localization

The 5′ end of *CpCpn60* contains a putative mitochondrial pre-sequence with a cleavage site between aa 38 and aa 39, as predicted by the MitoProtII ver. 0a4 program (http://www.mips.biochem.mpg.de/cgi-bin/proj/medgen/mitofilter; Claros and Vincens 1996) This site is not consistent with the rule of arginine at −2. However, not all targeting sequences follow the rules for cleavage sites (Taylor et al. 2001). The targeting sequence was predicted by the iPSORT program, which uses the aa index with alphabet indexing and pattern rules to determine the presence of a signal sequence. (See: http://www.hypothesiscreator.net/PSORT). Targeting was confirmed by MitoProt II, which predicts both the probability of mitochondrial import and cleavage sites. Since transfection of *Cryptosporidium* has not been reported, a yeast mitochondrial signal sequence ligated to GFP was replaced by the *C. parvum* Cpn60 putative targeting sequence to determine whether it would properly localize to mitochondria in a heterologous system. To be certain that the entire pre-sequence was included in the vector, the first 170 bp (57 aa) of *CpCpn60* (Fig. 1, horizontal arrows) were generated by PCR with *Eco*RI and *Bam*HI linkers on either end of the sequence. When the chimeric *CpCpn60*/GFP vector (pCpn60-57-GFP) was transfected into yeast cells, GFP correctly targeted the yeast mitochondrial network (Fig. 4A). This pattern of fluorescence was identical to that seen in positive control cells containing the *N. crassa* Su9 mitochondrial pre-sequence (pSu9-69-GFP) known to target the branched tubular network of the *S. cerevisiae* mitochondrion (Fig. 4E; Westermann and Neupert 2000). The specificity of this localization was confirmed when these cells were treated with 05 nM MitoTracker red, a known mitochondrion-specific vital fluorescent dye (Fig. 4B, F). Merged images (Fig. 4, orange) clearly show co-localization of these two fluorescent dyes to the yeast mitochondrial network (Fig. 4D, H). Vectors without a pre-sequence showed a diffuse staining pattern throughout the yeast cells (data not shown). These data clearly demonstrate that the *CpCpn60* pre-sequence can correctly target a protein to a mitochondrion.

Affinity-purified antibodies generated against *CpCpn60* peptides corresponding to aa 67–84 and aa 230–248 and conjugated to mcKLH were tested by Western blot. The immune serum reacted with a band at approximately 66 kDa as predicted (Fig. 2C, left panel) and shows no cross-reactivity with pre-immune sera

Fig. 2A–C Southern blot, RT-PCR, and Western analysis of CpCpn60. **A** Southern blot of *CpCpn60* using gDNA digested with *Eco*RI, *Hin*dIII, *Eco*RI/*Hpa*III, or *Eco*RV, respectively. Standard molecular masses are *designated on the left margin*. **B** RT-PCR of *CpCpn60* using total *C. parvum* RNA isolated from normal (*Uninf*., *lanes 3, 4*) or *C. parvum*-infected HCT-8 cells (*Cp Inf*., *lanes 5, 6*) and from isolated sporozoites (*Cp Spz*., *lanes 7, 8*). *Lanes 1, 2* contain RNA and primers from the kit (positive control) with and without reverse transcriptase, respectively. Amplicons were visualized on 1.0% agarose gels. **C** Western blot of native CpCpn60 from lysed sporozoites using affinity-purified rabbit antibody to the *C. parvum* Cpn60 peptide. Immune serum detects a single parasite protein at 65.6 kDa (*left panel*), whereas no parasite proteins are detected using pre-immune serum (*right panel*)



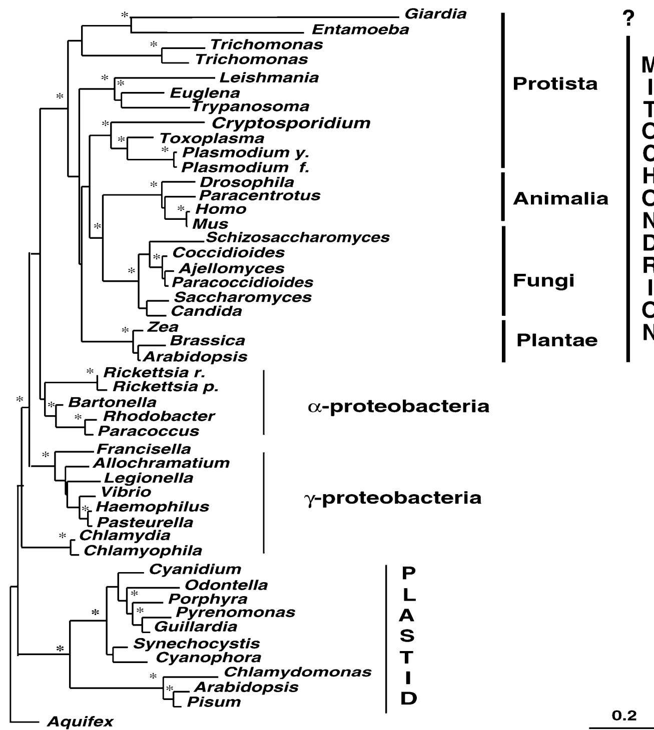


Fig. 3 Phylogenetic relationship of 47 Cpn60 amino acid sequences. The rooted phylogram was constructed with a maximum likelihood algorithm implemented in the PROTML ver. 2.3 program, using the Jones–Taylor–Thornton model with amino acid frequencies and alpha values of the gamma distribution estimated from data based on a neighbor-joining topology. *The 0.2 scale* denotes 20% amino acid substitutions per site. *Asterisks* indicate >85% bootstrap proportions

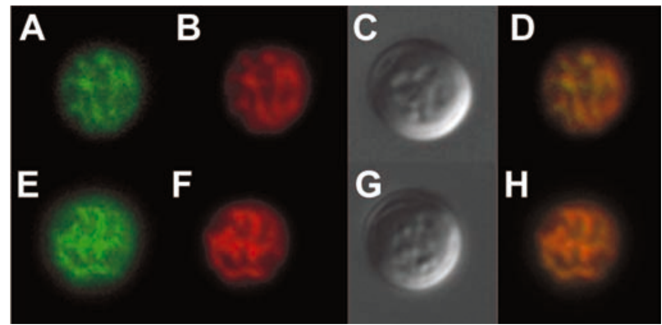


Fig. 4A–H The N-terminal peptides of *CpCpn60* target green fluorescent protein (GFP) to the *Saccharomyces cerevisiae* mitochondrial network. The mitochondrion-targeted plasmid pYX223-mtGFP, which contains a known mitochondrial presequence from protein 9 of the F0 part of the F0F1 ATPase of *Neurospora crassa* (Su9) and is regulated by the GAL promoter was constructed to show the intracellular distribution of *C. parvum* chaperonin 60 fusion proteins in transfected yeast. All micrographs are magnified ×1,000. *Bar* 10 μm. **A–D** show the mitochondrial network of yeast cells: **A** transfected with pCpn60-57-GFP vector, **B** exposed to MitoTracker red, **C** visualized by differential interference contrast, and **D** merged GFP/MitoTracker red fluorescent images confirming specific colocalization to the tubular network of the *S. cerevisiae* mitochondrion. **E–H** are identical images, except that the yeast targeting signal pSu9-43-GFP was used as a positive control. Both pCpn60-57-GFP and pSu9-69-GFP fusion proteins co-localized with the mitochondrial marker MitoTracker (**D**, **H**). The GFP vector without a targeting signal was diffusely distributed and showed no mitochondrial localization (data not shown)

(right panel). FITC-conjugated antibodies to CpCpn60 localized to the posterior end of sporozoites (data not shown) but, because sporozoites are very small (1–3 μm by 6–9 μm), localization within a specific organelle could not be determined.

Because ultrastructural studies had previously revealed a putative relict mitochondrion posterior to the nucleus in sporozoites (Riordan et al. 1999), the localization of CpCpn60 was tested by immunoelectron microscopy. A representative freshly excysted sporozoite fixed in formaldehyde/glutaraldehyde, embedded in LR White (which does not delineate membranes), and subsequently exposed to affinity-purified antibody raised against CpCpn60 clearly shows specific localization of immunogold particles to the organelle located between the nucleus and the crystalloid body (Fig. 5A, boxed enlargement). Using Student′s *t*-test ($t = 2.90 \pm 2.24$, 57 degrees of freedom), these results were statistically significant ($P < 0.005$; Fig. 5B). Analysis of the negative controls (antibody + peptide) showed no significant localization ($P < 0.81$) to sporozoite cytoplasm, membranes, or other subcellular organelles (Fig. 5A, B). As expected, the positive control showed diffuse labeling of all *C. parvum* sporozoite membranes (data not shown). Further evidence that this structure is a relict mitochondrion is demonstrated in Fig. 6. Here, freshly-excysted sporozoites were initially fixed in buffered 2% gluteraldehyde, with additional fixation in 2% osmium tetroxide and 0.5% uranyl acetate, and then embedded in epoxy resin to delineate ultrastructural membranes. This micrograph clearly shows an organelle (150–300 nm) enveloped in rough endoplasmic reticulum (RER), double-membrane-bounded, posterior to the nucleus, and in close apposition to the crystalloid body. It clearly shows this organelle is identical to the structure specifically labeled with CpCpn60 immunogold particles and observed in Fig. 5.

Discussion

Here, we show by transmission electron microscopy that the apicomplexan *C. parvum*, like these protists, possesses a double-membrane-bounded, RER-enveloped relict organelle posterior to the nucleus in close apposition to the crystalloid body (Fig. 6). Both sequence and phylogenetic analyses suggest that CpCpn60 shares a common ancestry from an α-proteobacterium. Unlike other heat-shock proteins, which may be organellar and/or cytosolic, these data are consistent with the observation that most known protist chaperonin 60s belong to Group I, which includes those of bacteria and eukaryotic mitochondria or plastids (Van der Giezen et al. 2003). Although we cannot eliminate the possibility that a cytosolic Cpn60 might be discovered in one or more apicomplexan genome projects, to date Group II archeal or eukaryotic cytosolic Cpn60 have been con-

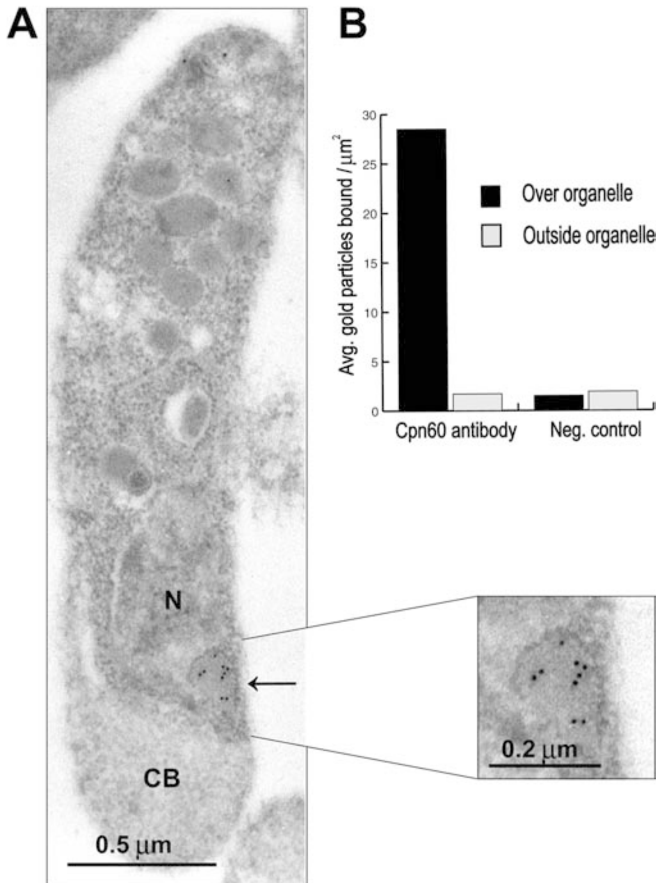


Fig. 5A, B Transmission electron microscopy immunogold localization of CpCpn60 within *C. parvum* sporozoites. **A** Representative longitudinal section of a LR White-embedded sporozoite shows nine CpCpn60-specific immunogold particles labeling the organelle posterior to the nucleus and in close apposition to the crystalloid body. A boxed enlargement of the posterior region containing the organelle *is shown at right*, and this corresponds to the osmium-fixed double-membrane-bounded structure wrapped in ribosomes seen in Fig. 6. There are three randomly distributed immunogold particles at the apical end. **B** Statistical analysis of gold particles localized over the organelle shows 27 ± 2.24 CpCpn60-specific immunogold antibody particles bound per square micron versus 2.0 ± 1.5 randomly distributed over subcellular structures outside the organelle ($n = 34$ sporozoites). Negative controls were exposed to CpCpn60-specific antibody plus blocking peptide ($n = 24$ sporozoites). Student's *t*-test indicates that localization is significant ($P < 0.005$) over the organelle

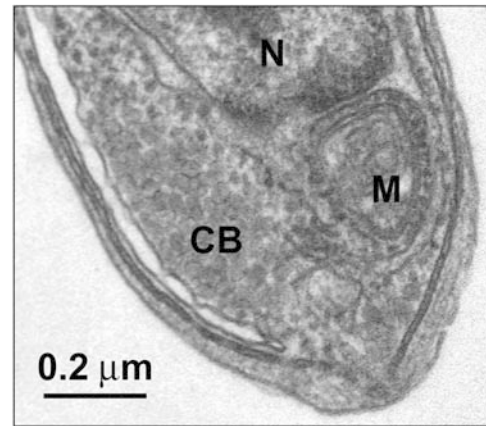


Fig. 6 Transmission electron micrograph of the *C. parvum* relict mitochondrion, showing location within sporozoites and relationship to other organelles and their membranes. A freshly-excysted sporozoite was initially fixed in buffered 2% gluteraldehyde, with additional fixation in 2% osmium tetroxide and 0.5% uranyl acetate. This fixation clearly shows organellar membranes. The relict mitochondrion is double-membrane-bounded, wrapped in rough endoplasmic reticulum, and contains internal cristae-like structures. Its location posterior to the nucleus and in close apposition to the crystalloid body confirms that it is the same organelle that is labeled in Fig. 5 with immunogold particles to CpCpn60. Reprinted from Riordan et al. (1999, Fig. 3) with permission

firmed only amongst the jakobid flagellates, a diverse group of mitochondriate and amitochondriate eukaryotes, the excavate taxa (Archibald et al. 2002).

Furthermore, the predicted *C. parvum* Cpn60 presequence correctly targets the mitochondrial network of the yeast *S. cerevisiae*; and immunogold-labeled antibodies generated against CpCpn60 specifically localize to a structure (Fig. 5) positioned exactly where the double-membrane-bounded organelle containing cristae-like compartments is observed (Fig. 6). Although the significance of the envelopment of the relict mitochondrion by RER is as yet unknown, a prominent coating of rat liver mitochondria by ribosomes has been proposed as evidence for co-translational translocation of proteins across mitochondrial membranes (Crowley and Payne 1998). Virtually nothing is known about the compartmentalization of core energy metabolism in *C. parvum*.

As mentioned previously, in *C. parvum*, extended glycolysis is thought to be the primary source of ATP with no contribution by oxidative phosphorylation (Crawford et al. 2003; Entrala and Mascaro 1997). Furthermore, we recently showed that *C. parvum* encodes at least six proteins essential for iron-sulfur cluster assembly in eukaryotic mitochondria (Lill and Kispal 2000; LaGier, MJ, Tachezy J, Kutisova K, Stejskal F, Keithly JS, unpublished data). Our working hypothesis is that iron-sulfur cluster biogenesis is one function of the *C. parvum* relict organelle. This is consistent with previous reports that genes for assembling mitochondrial-type iron-sulfur clusters occur in the protists *G. lamblia* and *T. vaginalis* (Tachezy et al. 2001) and the microsporidian *Encaphalitozoon cuniculi* (Katinka et al. 2001).

Among Apicomplexa, both *P. falciparum* and *C. parvum Cpn60* genes possess a single exon which yields one transcript (Syin and Goldman 1996; this study). These differ from the *Hsp60* of *T. gondii* (*TgHsp60*) which contains five introns and six exons, the splicing of which yields two distinct mRNAs (Toursel et al. 2000). Although both *T. gondii* Hsp60 transcripts are detected in both slow-growing bradyzoites and rapidly replicating tachyzoites, they are differentially enriched about 2-fold in the bradyzoites. Furthermore, immunofluorescent labeling indicated differential targeting of *T. gondii* Hsp60 (Toursel et al. 2000). Like *C. parvum* sporozoites, the single mitochondrion of tachyzoites was labeled, but rather unexpectedly the chaperonin targeted two un-

known vesicles in bradyzoites. Among the Alveolata, dual localization of Hsp60 has been noted for the ciliate *Tetrahymena thermophila* (Takeda et al. 2001). Here, the chaperonin is a bifunctional protein, localizing as a citrate synthase within mitochondria and as a 14-nm cytoskeletal filament within the microtubule organizing centers during cytokinesis. Whether *C. parvum* might also exhibit differential localization of CpCpn60 to structures other than the mitochondrion during its life cycle is not yet known.

The *CpCpn60* sequence also has some distinctive features. One of these is the absence of a commonly shared C-terminal GGM repeated motif (Fig. 1, overlines). As mentioned previously, this motif is also missing from the Cpn60 of the protists *Giardia*, *Entamoeba*, and *Leishmania* (data not shown) and from plastid/chloroplast Cpn60 (Hemmingsen et al. 1988) represented by the red alga, *Porphyra*. Among the Apicomplexa, *C. parvum* lacks a GGM motif, whereas both *Plasmodium falciparum* and *P. yoelii* have one motif each (Sanchez et al. 1999) and *Toxoplasma gondii* possesses four C-terminal GGM repeats (Toursel et al. 2000). These data indicate that the loss of this motif may have occurred after *Cryptosporidium* diverged from these other Apicomplexa; and they lend further support to the hypothesis that the genus *Cryptosporidium* constitutes an early emerging branch within the Apicomplexa (Zhu et al. 2000a).

The significance of this repeat is as yet unknown, since *Escherichia coli* can replicate at normal rates using a GGM-truncated form of GroEL, the bacterial homologue of Cpn60 (McLennan 1993). Some proteins lacking the GGM repeat instead possess multiple histidines, e.g. *Mycobacterium tuberculosis*; and organisms with many copies of Cpn60 frequently have at least one with a GGM motif (Karlin and Brocchieri 2000). Its conservation across diverse lineages, however, does suggest an essential function. Both *C. parvum* and *P. falciparum* encode C-terminal tails rich in serine or aspartic and glutamic acid residues, respectively. The significance (if any) of these tails is also unknown.

In summary, sequence and phylogenetic analyses indicate that *C. parvum* Cpn60 is a mitochondrial isotype and that antibodies generated against peptides from this protein localize to a double-membrane-bounded relict organelle posterior to the nucleus. These data confirm that this organelle, like the hydrogenosome of *Trichomonas vaginalis*, the mitosome (crypton) of *Entamoeba histolytica* and other "amitochondriate" protists, and mitochondrial remnants from microsporidians (Katinka et al. 2001; Williams et al. 2002), arose by a symbiogenic event from a common ancestor and that it is of mitochondrial origin.

Acknowledgements This work was supported in part by funds from National Institutes of Health grant AI40320 to J.S.K. and USUHS grants R073AM and R073IF to S.G.L. These data were submitted to the State University of New York-Albany in partial fulfillment for a PhD degree in Biomedical Sciences (C.E.R.) and have been presented in part at national and international meetings. At the Wadsworth Center, we thank the Electron Microscopy, Photo/Illustration, Molecular Genetics, and Peptide Synthesis Core Facilities, Fogarty Fellow Dr. J.R. Slapeta for assisting with GFP/MitoTracker red double-labeling yeast mitochondria, and members of the dissertation committee for many helpful comments. At Rockefeller University, we thank Dr. L.B. Sanchez for assistance with the phylogenetic analyses and Dr. M. Muller for critically reviewing the manuscript.

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