Cryptosporidium parvum Mitochondrial-Type HSP70 Targets Homologous and Heterologous Mitochondria

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A mitochondrial HSP70 gene (Cp-mtHSP70) is described for the apicomplexan Cryptosporidium parvum, an agent of diarrhea in humans and animals. Mitochondrial HSP70 is known to have been acquired from the proto-mitochondrial endosymbiont. The amino acid sequence of Cp-mtHSP70 shares common domains with mitochondrial and proteobacterial homologues, including 34 amino acids of an NH2-terminal mitochondrion-like targeting presequence. Phylogenetic reconstruction places Cp-mtHSP70 within the mitochondrial clade of HSP70 homologues. Using reverse transcription-PCR, Cp-mtHSP70 mRNA was observed in C. parvum intra-cellular stages cultured in HCT-8 cells. Polyclonal antibodies to Cp-mtHSP70 recognize a ~70-kDa protein in Western blot analysis of sporozoite extracts. Both fluorescein- and immunogold-labeled anti-Cp-mtHSP70 localize to a single mitochondrial compartment in close apposition to the nucleus. Furthermore, the NH2-terminal presequence of Cp-mtHSP70 can correctly target green fluorescent protein to the single mitochondrion of the apicomplexan Toxoplasma gondii and the mitochondrial network of the yeast Saccharomyces cerevisiae. When this presequence was truncated, the predicted amphiphilic α-helix was shown to be essential for import into the yeast mitochondrion. These data further support the presence of a secondarily reduced relict mitochondrion in C. parvum.

The apicomplexan parasite Cryptosporidium parvum infects humans and other animals worldwide, causing self-limiting disease in healthy hosts but life-threatening disease in immunocompromised individuals. Humans become infected with C. parvum when they ingest resistant oocysts in water, soil, or food. After ingestion, the oocyst releases four infectious sporozoites that give rise to intracellular (but extracytoplasmic), asexually multiplying merozoites within enterocytes of the small intestine. Finally, sexual development leads to the development of thin-walled oocysts that may excyst within an immunocompromised host and prolong infection or thick-walled oocysts that are expelled to the environment (15). Species of Cryptosporidium belong to the obligate parasitic phylum Apicomplexa, which includes other agents of medical and veterinary importance, i.e., toxoplasmosis (Toxoplasma gondii), malaria (Plasmodium spp.), babesiosis (Babesia microti), and avian coccidiosis (Eimeria spp.). The phylum is characterized by the presence of an apical complex. Unlike the case with other members of the Apicomplexa (Coccidia, Hematozoa, and Gregarina), morphological evidence for a mitochondrion in Cryptosporidium spp. has been limited (4, 7, 41, 48), and some thought the genus was amitochondriate (15, 44). However, recent data indicate that the Cpn60-containing, ribosome-studied organelle of C. parvum is indeed a mitochondrial remnant (40).

The mitochondrion is a unique organelle of eukaryotes, the acquisition of which is explained by endosymbiosis of an α-proteobacterial proto-mitochondrion into the host cell (9, 32). Improved phylogenetic analyses, as well as the discovery of mitochondrial homologues in Giardia, Entamoeba, parabasalia, and microsporidia (22, 38, 47, 50), suggest that there are no ancestrally branching, amitochondriate eukaryotes, i.e., that the mitochondrial compartment is essential. According to robust morphological evidence and phylogenetic reconstruction, the common ancestor for all members of the Apicomplexa (Alveolata), including species of Cryptosporidium, must have possessed a fully functional mitochondrion. Among extant Apicomplexa, Cryptosporidium spp. are unique in the apparent absence of a well-developed and respiring mitochondrion.

Like other eukaryotes, most apicomplexans possess a mitochondrial genome, but the majority of mitochondrial proteins are encoded by the nucleus, synthesized as precursors in the cytosol, and imported into mitochondria (19, 29, 31). Unlike its apicomplexan relatives, C. parvum appears to lack both a mitochondrial genome (C. E. Riordan and J. Keithly, unpublished data) and apicoplast DNA (52). For example, Plasmodium falciparum has a 23-Mb nuclear genome that encodes 5,282 proteins, 466 of which (8.8%) are predicted to target the apicoplast (18) and 246 (4.7%) of which are predicted to target the mitochondrion (21). Initial analyses of the partial C. parvum nuclear genome indicate a putative existence of several nucleus-encoded mitochondrial proteins, but the existence of nucleus-encoded apicoplast proteins remains elusive (6).

The majority of mitochondrial matrix proteins have an NH2-terminal mitochondrial presequence that is removed by a specific matrix protease upon translocation across the mitochondrial membrane. Although presequences do not share a common primary amino acid structure, they all do have the ability to form a positively charged amphiphilic α-helix (36). Moreover, the binding of the presequence helix into an apolar groove in Tom20 chiefly depends upon hydrophobic, rather than hydrophilic, amino acids (1, 39). Although mitochondrial protein import is thought to be evolutionarily conserved in

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animals and plants, some differences exist. For example, plant mitochondrial targeting presences are longer and have a higher serine content (13). Because plants also contain nucleus-encoded genes for chloroplast proteins, it is thought that a more stringent organellar targeting system occurs in them and that programs analyzing plant genomes are subsequently less accurate in predicting mitochondrial presences (30).

Ubiquitous chaperones belonging to the 70-kDa class are known to bind immature proteins or preproteins and to facilitate their maturation and translocation across membranes into several subcellular compartments. Eukaryotes possess at least three types. Those of the cytosol and endoplasmic reticulum (ER) result from an ancient gene duplication in eukaryotic lineages, whereas that of the mitochondrion results from endosymbiosis of a DNA-containing α-proteobacterium together with numerous other mitochondrial proteins (8, 29).

The mitochondrial 70-kDa chaperone (mtHSP70) is an essential component of the Tim (translocase of the inner membrane) mitochondrial import complex, which binds preproteins on the matrix side of the inner mitochondrial membrane, and serves as an import motor for matrix proteins (39). Although a cytosolic HSP70 had been known for C. parvum (26), neither an mtHSP70 nor a component of the mitochondrial translocase machinery had been observed.

Here we describe a C. parvum mtHSP70 (Cp-mtHSP70) that (i) is a nuclear gene with clear proto-mitochondrial origins, (ii) possesses a mitochondrial targeting sequence, and (iii) is part of the mitochondrial protein import machinery. A reporter gene (green fluorescent protein [GFP]) is used to show that the C. parvum presequence targets mitochondria in the apicomplexan T. gondii and the yeast Saccharomyces cerevisiae. Analyses of truncated C. parvum targetig sequences revealed the amino acids within the predicted helix of the presequence to be essential for import. Fluorescence and immunogold microscopy showed the localization of labeled polyclonal antibody to be essential for import. Fluorescence and immunogold microscopy showed the localization of labeled polyclonal antibody to be essential for import. Fluorescence and immunogold microscopy showed the localization of labeled polyclonal antibody to be essential for import.

**MATERIALS AND METHODS**

**Parasites and cell cultures.** Cryptosporidium parvum (Iowa strain; bovine origin) oocysts were obtained and treated as previously described (51). Human infected cultures were treated identically, except that C. parvum predicted gene was amplified using primers specific for C. parvum small subunit (SSU) ribosomal DNA and human β-globin. The cDNA was synthesized from total RNA using random hexamer primers from the SuperScript First-Strand Synthesis system for RT-PCR (Invitrogen). Subsequent PCR with 1 U of TaqDNA polymerase (Promega) containing 25 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl2, 250 μM dNTPs, and 0.5 μM of each primer was performed (25°C for 45 s, 94°C for 1 min, 35 cycles). Product purity was confirmed by staining on a 3% agarose gel. PCR products were purified and cloned in frame, using EcoRI and HindIII-digested pYX122. As a positive control, a vector overexpressing only GFP (with no ability to target mitochondria) was used. The identity of the vectors was verified by sequencing. Standard methods for yeast growth and transformation were used (49). Yeast cultures grown in selective media were examined with a Zeiss Axioskop 2 motplus fluorescence microscope. The MX1-4C yeast strain was a gift from R. Morse (34).

**Generation of the T. gondii construct and transfection.** To evaluate the ability of the Cp-mtHSP70 presequence to target the intracellular compartment of T. gondii tachyzoites, the N-terminal 46 amino acids (MSM1SSSNF GVNVNSSGAA RILRKLSPLV FSRYSMSSKE GKSSNNWW) were introduced in-frame between GRA1 and GFP using gene splicing by overlap extension (11). The HindIII-NsiI construct was generated using a fusion GRA1 plus mtHSP70 primer (5′-ACT ATT ATAT CAT AGA CAT CCT GCT TAA TT CAA AGT-3′). The amplified product was digested with HindIII and NsiI and cloned into the mitoG1/2-GFP vector (with no ability to target mitochondria) by an XbaI restriction site, using primers with XbaI and NotI, and these were cloned into respective expression vectors. The transformants were selected on 25 μg/mL G418 and 40 μg/mL potassium acetate (KA) medium, and were identified by RT-PCR and semiquantitative RT-PCR of Cp-mtHSP70 in cDNA, the forward primer (5′-AGG TTT CTT TGG AAG GTA GTA GAG-3′) and reverse primer (5′-CTT GGA GTT TCA AAG TTA GCT GG-3′). Amplification was performed using a GeneAmp 9700 (Perkin-Elmer) cycler as follows: initial denaturation at 95°C for 5 min, cycling at 94°C for 30 s, 55°C for 30 s, 74°C for 60 s (total of 24 and 30 cycles), and a final extension at 74°C for 10 min. Amplicons were separated on 5% nondenaturing acrylamide gels and were visualized by phosphor-imaging (STORM8600) using ImageQuant 5.1 software (Molecular Dynamics). For identification of Cp-mtHSP70 in cDNA, the forward primer (5′-ATG CTT GGG TAG AAG CTA GAG-3′) and reverse primer (5′-GAA GTG TTA CCA TTA GTT GC-3′) yielded a 360-bp amplicon. To determine the ratio of C. parvum signal to growing parasites in infected cultures, the mRNA signal (cDNA dilution 1:50) was normalized to the C. parvum RNA signal derived in each of the independent experiments, which were then analyzed. Controls of RNA isolated from parallel cell cultures were used as a control, a vector overexpressing only GFP (with no ability to target mitochondria) was used. The identity of the vectors was verified by sequencing. Standard methods for yeast growth and transformation were used (49). Yeast cultures grown in selective media were examined with a Zeiss Axioskop 2 motplus fluorescence microscope. The MX1-4C yeast strain was a gift from R. Morse (34).

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washed once overnight in 50 ml of H₂O and once overnight in 50 ml of PBS (pH = 7.4) and then homogenized in a final volume of 1 ml of PBS (pH = 7.4) and injected into a female rabbit (Y910) at 2-week intervals. Preimmune serum was bled prior to the first dose. The total serum of this rabbit was purified 2 weeks after and tested on a Sulfolink gel column coupled with MRP-Cp-mtHsp70/101-34 using a Sulfolink kit (Pierce). For Western analysis, a working dilution of 1:1,000 was used for purified anti-Cp-mtHSP70.

Western analyses and measurement of the import of GFP. Yeast mitochondrial extracts were prepared from yeast spheroplasts, and subcellular extracts (including purified mitochondria) were prepared as described by Diekert et al. (12). The final volumes of proteins from different extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to an Immun-Blot polyvinyl difluoride membrane (Bio-Rad). Nonspecific binding was blocked using 10% nonfat dry milk–3% bovine serum albumin (BSA) in PBS with 0.05% Tween-20 (PBS-T) for 1 h. Antibodies were diluted in 3% BSA–PBS-T and incubated at room temperature for 60 min. For detection of yeast GFP import, rabbit polyclonal antibodies and purified anti-GFP (Invitrogen) were used at a dilution of 1:5,000. Mouse monoclonal anti-yeast cytochrome oxidase subunit III (DA3) was used at a concentration of 2.5 μg/ml (Molecular Probes). Secondary goat anti-rabbit horseradish peroxidase conjugate (1:10,000; BioSource) and goat anti-mouse horseradish peroxidase conjugate (1:2,500, Promega), respectively, were incubated for 60 min. Results were analyzed by using Chemiluminescence Reagent Plus (PN: 170,500; NEN). The signals were quantified using ImageQuant 5.1 (Molecular Dynamics). The GFP signal was detected for mitochondrial and cytosolic fractions prepared as described by Diekert et al. (12). To take into account the variability in the fragmentation of mitochondria during homogenization, values were corrected by the ratio obtained for the endogenous mitochondrial cytochrome oxidase subunit III. For each construction, GFP import was expressed as a ratio between GFP detected in the prepared mitochondrial fraction to the cytosolic and mitochondrial fraction together. The resulting number was then expressed as a percentage of the ratio obtained for the yeast SSC1 control ± standard deviation.

Immunofluorescent antibody staining. All manipulations were carried out at room temperature. Freshly excysted C. parvum sporozoites attached to poly-L-lysine–coated slides and in vivo-cultured intracellular parasite stages in HCT-8 cells attached to the Transwell (Costar) membranes were washed with PBS (pH = 7.4), fixed in fresh 4% paraformaldehyde in PBS for 5 min, and rinsed in 0.1 M glycine–PBS. Cells were permeabilized using 0.2% Triton X-100 in 3% BSA–PBS-T for 3 min and were then washed with PBS. Slides were incubated for 120 min with purified anti-Cp-mtHSP70 antibodies in 3% BSA–PBS-T (dilution, 1:50), washed, and incubated further for 60 min in 1:50 green fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit immunoglobulin G (Sigma) diluted in 3% BSA–PBS-T. The slides were counterstained for 5 min with 0.1 μg of the blue fluorescent nuclear stain DAPI (Sigma) ml⁻¹. Finally, slides were mounted using the SlowFade Light Antifade kit (Molecular Probes). The slides were examined under a Zeiss Axioskop 2 mot microscope; black–white and fluorescent images were recorded with the ORCA-ER (Hamamatsu) digital camera and then superimposed and pseudo-colored by using OpenLab 3.1. software (Improvision). Controls were processed in parallel. These included preimmune serum as the primary antibody, with those without primary antibody containing only secondary antibodies.

Electron microscopy. Purified sporozoites were fixed and embedded in epoxy or LR White as previously reported (40, 41). Briefly, sporozoites for epon embedding were fixed in osmium and electron microscopic (EM)-grade glutaraldehyde, whereas those for LR White were fixed in EM-grade melanin-free formaldehyde, with additional fixation in 4% formaldehyde–0.1% EM-grade glutaraldehyde. Thin LR White sections (0.1 μm) were incubated with purified anti-Cp-mtHSP70 or a preimmune serum as a primary antibody and a 1:50 dilution of 10-nm-gold-conjugated goat anti-rabbit immunoglobulin G as a secondary antibody and examined under a Zeiss electron microscope.

Prediction analyses. Plant and nonplant algorithms were used with TargetP1.0 to predict protein localizations (14). Apicoplast targeting was predicted using PlasmoAP (18). Secondary structures were predicted using SSProB, PsiPred, and Predator (42).

Phylogenetic analyses. Multiple sequence alignments included 53 sequences from DnaK and eukaryotic homologues. Initially the National Center for Biotechnology Information database was searched (blastp), using DnaK and its known homologues to retrieve a broad spectrum of sequences (3). To determine the origin of Cp-mtHSP70, only a limited number of diverse representatives from bacterial and eukaryotic taxa were finally selected because of the extensive computational analyses required. The existing homologues ofT. gonditi and T. cruzi mitochondrial HSP70 were not used in the final phylogenetic analyses due to their origin within the unfinished T. gonditi genome project. Sequences were aligned using Clustal X 1.81, PAM 250 matrix (45), and those ambiguously aligned were excluded to finally yield a total of 398 residues. These are available from the authors upon request.

Two methods were used for tree reconstruction based on amino acid sequence alignment. Protein maximum-likelihood analysis employing the JTT model of amino acid evolution (alpha parameter = 2, with eight rate categories of amino acid changes) and bootstrapped using 100 replicates was performed with ProML, ScqBoot, and Consense programs of PHYLIP 3.6a3 (16). A Bayesian phylogenetic search for tree space used a variant of the Markov chain Monte Carlo and was performed in the MrBayes 3.01 program (24). Metropolis-coupled Markov chain Monte Carlo analysis used the JTT model of amino acid evolution. The Markov chain was started from a random tree and run for 500,000 generations, sampled every 100 generations with four chains. The first 30,000 generations were finally discarded for calculation of posterior probabilities (PP).

Nucleotide sequence accession number. The complete sequence for Cp-mtHSP70 has been deposited in the GenBank database under accession no. AY235430, along with the conceptually translated peptide under accession no. AAP59793.

RESULTS

C. parvum possesses mtHSP70. A 2,052-bp intronless gene encoding a protein of 683 amino acids with a predicted molecular mass of 74.6 kDa (excluding the targeting presequence, 70.9 kDa) and having significant homology to mitochondrial homologues of the 70-kDa heat shock protein family (HSP70) was cloned and sequenced from C. parvum (Cp-mtHSP70). The AT content is 62.8%. A BLAST (blastp) search yielded highest scores with eukaryotic mitochondrial HSP70 homologues and prokaryotic DnaK. The sequence of Cp-mtHSP70 (accession no. AAP59793) contains 46% identities, 64% conservative substitutions, and 3% gaps compared (BLAST2) to C. parvum cytosolic HSP70 (accession no. AAC25925) (26). Unlike the case with cytosolic HSP70, no amino acid repeats of GGMP were found near the COOH terminus of Cp-mtHSP70. The identity (similarity) scores for Cp-mtHSP70 and HSP70 from completely sequenced genomes (P. falciparum and S. cerevisiae) using BLOSUM62 are as follows: for mitochondrial P. falciparum, 69% (79%) sequences, and for S. cerevisiae, 55% (72%) sequences; for cytosolic P. falciparum, 40% (58%) sequences, and for S. cerevisiae, 42% (59%) sequences. The amino acid sequence analyses indicate that Cp-mtHSP70 is a mitochondrial-type HSP70 (Fig. 1).

Total RNA from C. parvum-infected HCT-8 cells isolated at 6, 12, 24, and 72 h postinfection (p.i.) and from freshly excysted and purified sporozoites was analyzed by semiquantitative RT-PCR to determine whether Cp-mtHSP70 is differentially expressed. Amplification through 24 cycles using specific primers yielded a distinct 360-bp RT-PCR product in HCT-8 cells at all h p.i., as well as in sporozoites (Fig. 2A). The overall pattern of amplification was identical in three independent experiments. Minor differences were noted in product abundance: peaks were highest at 12 and 72 h p.i. and decreased at 48 h p.i. (Fig. 2B). Although the signal for sporozoites is greater than those for all intracellular time points, interpretation is limited by the fact that these samples were prepared independently from those of HCT-8 cultures. Furthermore, and in congruence with previous data (2), C. parvum-infected HCT-8 controls began transcribing actin 12 h p.i., which gradually decreased over time, whereas transcription of the Cryptosporidium oocyst wall protein occurred during gametocyte (sexual) development at 48 to 72 h p.i. (data not shown). Together, these results suggest
FIG. 1. Multiple protein sequence alignment of cytosolic and mitochondrial HSP70. Conceptually translated full-length amino acid sequences of *C. parvum* together with *S. cerevisiae* and *P. falciparum* were aligned using the program Clustal X and the BLOSUM protein weight matrix. The upper three sequences are mitochondrial; the lower three are cytosolic. Black shading indicates that identical amino acids are conserved, whereas gray indicates conservation of similar amino acids. The threshold for shading was set to 50%. A solid line denotes the predicted Cp-mtHSP70 mitochondrial presequence; a dashed underline denotes the SSC1 yeast mitochondrial presequence (43). The two mitochondrial/proteobacterial sequence signatures motifs, GDAWV and YSPSQI, are denoted with an asterisk above the alignment. The species name and GenBank accession numbers are indicated at the right of the alignment.
that Cp-mtHSP70 is constitutively expressed during the life cycle.

**Phylogeny of mtHSP70 and DnaK homologues.** For initial analyses, databases were searched (blastp), using known HSP70 and DnaK homologues to retrieve sequences over a wide taxonomic spectrum (3). To determine the origin of Cp-mtHSP70, 53 sequences from representative bacterial groups and eukaryotic taxa were selected (Fig. 3). The alignment consisted of 398 residues. Protein maximum-likelihood analysis with bootstrapping replicates (BP) and a Bayesian phylogenetic search with the PP were calculated (see Materials and Methods). Cytosolic HSP70 types are clearly monophyletic, with a PP value of 1.00 and BP value of 82%. The resolution within the ER HSP70 cluster is imprecise due to the less reliable alignment for them, but overall ER types of HSP70 are a sister group to those of the cytosol. The monophyly of cytosolic and ER HSP70 is supported with 1.00 PP and 100% BP. Mitochondrial HSP70 types cluster within /H9251-/H9251-proteobacterial DnaKs and the other bacteria as a monophyletic mitochondrial branch. Although the best-reconstructed trees support the monophyly of the mitochondrial branch, there is only 0.69 PP and <50% BP support. The sister relationship of /H9251-/H9251-proteobacterial sequences and the mitochondrial clade is also less supported (0.63 PP and <50% BP). This is due to an alternative branching of *Erlacha* plus *Rickettsia* and *Rhodopseudomonas* plus *Agrobacterium* plus *Sinorhizobium* with mitochondrial HSP70, i.e., maximum-likelihood monophyly of *Erlacha* plus *Rickettsia* with mitochondrial HSP70. The topology of the reconstructed trees is essentially identical to those recently reported for the analyses of homologues from *Giardia*, *Entamoeba*, parabasalia, and microsporidia (5, 22, 35).

Because the full genome sequence for *P. falciparum* is known (21), all available sequences for its HSP70 complex have been retrieved. There is a single copy each of cytosolic, ER, and mitochondrial HSP70 in the genome of *P. falciparum*. As expected, phylogenetic reconstruction of *P. falciparum*, *C. parvum*, and other apicomplexan sequences shows that they cluster together (Fig. 3). Mitochondrial homologues from *C. parvum*, *P. falciparum*, and *Eimeria tenella* form a monophyletic clade within eukaryotic mitochondrial DnaK homologues, whereas cytosolic HSP70 types of *C. parvum*, *P. falciparum*, and *Theileria* plus *Babesia* are monophyletic within the eukaryotic cytosolic HSP70 clade (Fig. 3). This monophyly is strongly supported (1.00 PP and 99 to 100% BP). Thus, the phylogenetic distinctions between apicomplexan cytosolic and mitochondrial HSP70 strongly suggest different evolutionary origins for them: cytosolic HSP70 from a common ancestor with eubacteria and archaea, and mitochondrial HSP70 from an endosymbiotic event. Moreover, the ER type (also known as BiP) required to power ER posttranslational translocation by pulling the polypeptide into the ER membrane (8) has been identified in the completed *C. parvum* genome (www.CryptoDB.org).

**Presequence of Cp-mtHSP70.** TargetP was used to predict the in silico localization (Table 1) of apicomplexan and yeast protein sequences that cluster within the mitochondrial and cytosolic branches of the phylogenetic tree, i.e., *P. falciparum*, *S. cerevisiae*, and *C. parvum* (Fig. 3). Although both plant and nonplant algorithms were used, a specific organelar compartment for the putative *P. falciparum* mtHSP70 homologue could not be assigned. For Cp-mtHSP70, on the other hand, the nonplant algorithm predicts a mitochondrial localization (0.74), but using a plant algorithm, compartmentalization into a chloroplast or apicoplast (0.79) instead of a mitochondrion (0.05) is predicted. PlasmoAP was used to further test the potential of
Cp-mtHSP70 sequences for apicoplast targeting (18). No signal sequence of the bipartite apicoplast targeting presequence was detected, and apicoplast targeting was rejected.

Based upon SSPro8, Psi-Pred, and Predator programs for predicting secondary structures, the C. parvum presequence was predicted to form α-helices among amino acid residues 15 to 26, 17 to 38, and 15 to 29 (Fig. 4A). Using Eisenberg’s plot, a significant hydrophobic moment was observed for residues 15...
to 26 (Fig. 4A), suggesting the possibility of a bifacial amphiphilic α-helix between these amino acids (Fig. 4B).

Several motifs are known to be specific cleavage sites for a mitochondrial protein peptidase (20): motif R-2 \( \text{xRx}^2 \text{xx(S/x)} \); motif R-3 \( \text{xRx(Y/x)}^2 \text{xx(S/A/x)x} \); or R-none motif \( \text{xx}^2 \text{xx(S/x)} \).

The primary amino acid sequence of Cp-mtHSP70 contains an R-2 motif, \( 31\text{FSR}Y^2\text{MSK}38 \) (signature amino acids in bold), and predicts a mitochondrial presequence of 34 amino acids (signature amino acids in bold).

Although there are two potential initiation codons at the 5' end of this peptide, based upon consensus nucleotide start codon sequences, the first ATG was chosen as the initial start of translation.

Heterologous targeting of Cp-mtHSP70 presequence to the mitochondrion of \( S. \) cerevisiae and \( T. \) gondii.

To compare the in silico predictions for the \( C. \) parvum presequence with in vivo targeting to heterologous mitochondria, the presequence was tested for its ability to deliver GFP into the mitochondrial network of yeast and the genetically well-characterized apicomplexan \( T. \) gondii. Initially, the entire Cp-mtHSP70 presequence was cloned onto the NH2-terminal end of GFP in the yeast expression vector pYX122(mtHSP)GFP (Fig. 4C). The tubular mitochondrial network of pYX122(mtHSP)GFP-transformed yeast cells exhibited a high-intensity green fluorescence (Fig. 5A, Cp) that was identical to that of the control yeast mtHSP70 homologue, SSC1 (Fig. 5A, Sc). These data indicate that the complete Cp-mtHSP70 presequence is capable of delivering GFP into the mitochondrial network of heterologous mitochondria. The GFP import was quantified by Western blot analysis of mitochondrial and cytosolic fractions from transformed yeast. The variability of fragmentation of mitochondria

TABLE 1. Summary of in silico predicted protein localizations

| Acc. no. | Prediction | RC | mTP | cTP | SP | Other | Loc. |
|----------|------------|----|-----|-----|----|-------|------|
| Cp AAP59793 | mit 0.74/0.50 | 0.79 | 0.02/0.13 | 0.40/0.47 | M/C | | 4/2 |
| Cp AAC25925 | cyt 0.041/0.09 | 0.23 | 0.11/0.12 | 0.92/0.81 | 1/3 |
| Pt NP_701211 | mit 0.41/0.05 | 0.27 | 0.03/0.19 | 0.76/0.40 | 4/5 |
| Pt NP_704366 | cyt 0.11/0.09 | 0.16 | 0.06/0.16 | 0.89/0.74 | 2/3 |
| Sc AAA63792 | mit 0.93/0.62 | 0.23 | 0.01/0.04 | 0.15/0.28 | M/M | | 2/4 |
| Sc AAC37398 | cyt 0.15/0.16 | 0.10 | 0.07/0.21 | 0.15/0.72 | 2/3 |

Values were calculated by TargetP 1.0 using nonplant/plant algorithms; mTP and cTP, mitochondrial and chloroplast targeting peptides; SP, signal peptide; other, cytosolic localization or undetermined; Loc, localization based on "winner takes all" (either M = mitochondrion or C = chloroplast); RC, confidence index (1 = excellent; > 5 = poor); mit, mitochondrion homologue; cyt, cytosolic based on phylogenetic analyses. Acc. no., accession number of protein sequence. Organism is indicated at left of accession no.: Cp, \( C. \) parvum; Pt, \( P. \) falciparum; Sc, \( S. \) cerevisiae.

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FIG. 4. Presequence of \( C. \) parvum mtHSP70. (A) Secondary structure prediction. Line 1 (SSpro8): H, alpha-helix; G, 310-helix; I, pi-helix; E, extended strand; T, turn; S, bend; C, the rest. Line 2 (Psi-Pred): H, helix; E, strand; C, coil. Line 3 (Predator): H, helix; E, extended sheet; _, coil. Line 4: amino acid sequence of the Cp-mtHSP70 NH2 terminus. Eisenberg’s hydrophobic moment plot is immediately beneath and aligned with the amino acid sequence above it. The arrow indicates the R-2 motif, \( \text{xRx}^2 \text{xx(S/x)} \). Numbering denotes amino acid residues. (B) Helical wheel plot of residues 15 to 32 with putative amphiphilic α-helix demonstrating partitioning of charged and hydrophobic amino acids (gray and black circles, respectively). The helical wheel plot was generated with the aid of Java-applet http://ccti.itc.virginia.edu/~cmg/Demo/wheel/helwheelApp.html. (C) Truncated NH2-terminal amino acid sequences of Cp-mtHSP70 cloned into the \( E. \) coli/yeast shuttle vector pYX122-GFP, including the yeast SSC1 control (sequence in italic). Numbering above denotes amino acid residues. Constructs are named on the right. (D) Graph of the import efficiency of different GFP constructs based on Western blotting of subcellular fractions. Import efficiency is the ratio between mitochondrially imported GFP and total GFP (mitochondrial and cytosolic) relative to the control construct SSC1. Results are calculated from three independent experiments. Numbering of the constructs corresponds to that in panel 4C.
Cp-mtHSP70 getsing of GFP using the Cp-mtHSP70 presequence at the NH2 terminal regions of the presequence excised, are shown. Bar, 5 μm.

FIG. 5. Targeting of C. parvum mtHSP70 presequence to heterologous mitochondria. (A) Targeting of the yeast S. cerevisiae using the Cp-mtHSP70 presequence at the NH2 terminus of GFP vectors (see Fig. 4C). Each panel shows paired GFP and differential interference contrast (DIC) images. Full C. parvum presequence (Cp), negative control lacking the presequence (targeting the cytosol; cGFP), positive control—the yeast mtHSP70 homologue SSC1 presequence, targeting the yeast mitochondrial network (Sc), and the C. parvum truncated forms, Δ1-10, Δ1-20, Δ1-30, Δ10-20, Δ10-30, and Δ10-40, indicating the regions of the presequence excised, are shown. Bar, 5 μm. (B) Targeting of GFP using the Cp-mtHSP70 presequence at the NH2 terminus of GFP (upper panels, GRA1-[pre]-GFP) or no-presequence control (lower panels, GRA1-GFP) using transfected T. gondii RH cultured in human foreskin fibroblast cells. A released single tachyzoite is shown. The mitochondrion is also labeled with MitoTracker Red CM-H2XRos (MTR). In the upper panels, note targeting of GFP to the mitochondrion in the merged double-labeled image (merge). A composite of the merged image with DIC is shown on the right (DIC). Bar, 3 μm.

during homogenization and the release of GFP into the cytosolic fraction was corrected using immunodetection of the COXII mitochondrial marker. The complete C. parvum presequence delivered a GFP signal that was 96% ± 8% of that exhibited by isolated mitochondria of the SSC1 control. In contrast, the fluorescence of a negative control, vector Δ1-45(mtHSP)GFP lacking the presequence, diffusely stained the cytosol and not the mitochondrial network (Fig. 5A, cGFP). To further analyze the properties of the predicted helical structure of the presequence, truncated constructs were cloned into the pYX122-GFP vector (Fig. 4C). First, the targeting sequence was truncated consecutively at the NH2 terminus, Δ1-10, Δ1-20, and Δ1-30. Yeast transformed with the Δ1-10 and Δ1-20 constructs predominantly targeted the yeast mitochondrial network (Fig. 5A, Δ1-10 and Δ1-20), yielding a GFP fluorescence signal in the tubular mitochondrial network of 92% ± 1% and 79% ± 3%, respectively (Fig. 4D). The Δ1-30 construct showed a diffuse cytosolic fluorescence (Fig. 5A, Δ1-30) and only 5% ± 5% of the GFP signal was localized within the mitochondrion, i.e., the removal of 21 to 30 amino acids appears to remove targeting capability. Furthermore, when various internal parts of the presequence were truncated, but leaving the first 10 NH2-terminal amino acids in place, only the Δ11-20 construct was still able to specifically target the yeast mitochondrial tubular network (Fig. 5A, Δ11-20), delivering 75% ± 6% of GFP signal to the organelle. Constructs that eliminated most or the rest of the presequence showed a diffuse cytosolic fluorescence (Fig. 5A, Δ11-30 and Δ11-40), delivering only 13% ± 5% and 2% ± 2% GFP to the yeast mitochondrion, respectively (Fig. 4D). These data suggest that amino acid residues 11 to 30 are essential for mitochondrial targeting, with residues 21 to 30 being the most critical, while NH2-terminal residues 1 to 10 appear to be insignificant. The data essentially agree with the predicted importance of the bifacial amphiphilic α-helix in the C. parvum presequence (Fig. 4A).

Because of the in silico ambiguity for organellar targeting (Table 1), the genetically well-characterized apicomplexan T. gondii was tested by using the C. parvum complete mitochondrial presequence. T. gondii possesses both a mitochondrion and an apicoplast (17). As expected, tachyzoites transfected with pTgGRA1-pre-GFP (C. parvum presequence as an NH2 terminus of the GFP) correctly target the structurally well-defined T. gondii mitochondrion (33), whereas the plasmid lacking the C. parvum targeting presequence shows a diffuse cytosolic green fluorescence (Fig. 5B). Localization into the single T. gondii mitochondrion was confirmed by double labeling using the mitochondrion-specific dye MitoTracker Red CM-H2XRos (Fig. 5B, merge). This is the first time T. gondii has been used as a surrogate host for mitochondrial targeting of C. parvum sequences.

Cp-mtHSP70 localizes to the relict mitochondrion of C. parvum. To further investigate the expression of Cp-mtHSP70, a rabbit anti-Cp-mtHSP70 (see Materials and Methods) was tested by Western blotting. Polyclonal antibodies to a recombinant Cp-mtHSP70 recognized a single band in C. parvum extracts with an approximate molecular mass of 70 kDa (Fig. 2C).

Fluorescein (FITC)-labeled anti-Cp-mtHSP70 serum localized to a single compartment in freshly purified sporozoites of C. parvum (Fig. 6A), whereas preimmune control serum did not (data not shown). In sporozoites, the compartment localized by FITC-anti-Cp-mtHSP70 is in close apposition to the nucleus, as visualized by DAPI counterstaining, and the green
fluorescence always appears as a single ovoid spot posterior to the nucleus (Fig. 6A, arrowheads).

*Cryptosporidium parvum*-infected HCT-8 cells cultured on Transwell membranes were processed for immunofluorescence microscopy at 24, 48, and 72 h post-infection (h.p.i.) with anti-Cp-mtHSP70 (Fig. 6B). At 24 h p.i., most of the intracellular life cycle stages are meronts that contain four merozoites (Fig. 6B, 24 h). Each merozoite shows a distinct DAPI-stained nucleus next to which a FITC-immunofluorescent spot can be seen, and nearly all of the HCT-8 cells were infected with several meronts. At 48 h p.i., *C. parvum*...
developmental stages containing 4 to 8 nuclei, and adjacent FITC-labeled oval dots could be observed (Fig. 6B, 48 h). Some developmental stages at 48 h p.i. did not appear to have DAPI-stained dividing nuclei, although FITC-labeled organelles were still apparent. These parasites mostly resemble stages seen at 72 h p.i. The 72-h p.i. developmental stages were larger and often contained a DAPI-stained sphere to which several FITC-anti-Cp-mtHSP70 ovals were attached (Fig. 6B, 72 h). These stages are probably gametocytes of *C. parvum* which will eventually produce zygotes upon fertilization and subsequently yield either thin- or thick-walled oocysts. Some developmental stages stained with FITC-anti-Cp-mtHSP70 indicated not only a single round compartment, as seen in *C. parvum* sporozoites, but also a smaller, less distinct spot (Fig. 6B, arrowheads). Such staining might represent multiplication of both the organism and the organelles. However, the explicit interpretation of these two compartments could not be clearly resolved by conventional microscopy.

Immunoelectron microscopy of sporozoites was also used to determine the subcellular localization of Cp-mtHSP70 (Fig. 7). Previously we have shown that the relict mitochondrion, to which chaperone CpCpn60 has been localized (40), is posterior to the central nucleus in close apposition to the crystallloid body. Here we further confirm the presence of a mitochondrion in *C. parvum* by showing a full-length sporozoite (Fig. 7A) fixed in osmium and glutaraldehyde and stained with uranyl acetate. The membranes of the apical organelles, including the micronemes, rhoptry, and dense granules, as well as those of the posterior nucleus, relict mitochondrion, and crystallloid body, are clearly delineated. Three formaldehyde- and glutaraldehyde-fixed sections (which do not reveal membranes) embedded in LR White (Fig. 7B) clearly show the localization of 10-nm Cp-mtHSP70-specific immunogold particles to an organelle posterior to the nucleus and next to the crystallloid body. This compartment corresponds both to the relict mitochondrion observed in the epon-embedded section (Fig. 7A) and to that previously described for CpCpn60 (40).

**DISCUSSION**

The 70-kDa heat shock protein family is one that assists in protein folding in a variety of cellular compartments and that is broadly conserved among prokaryotes and eukaryotes. The HSP70 proteins consist of a highly conserved NH2-terminal domain and a C-terminal region subdivided into a 15-kDa conserved substrate-binding domain and a 10-kDa putative substrate-stabilization domain that is less well conserved (8). Although Cp-mtHSP70 has several regions with significant similarity to all HSP70 sequences, the greatest scores were obtained to those of proteobacterial and eukaryotic mitochondrial HSP70. Moreover, alignments confirm the presence of two sequence signatures (22) in Cp-mtHSP70: a 148-GDAWY152 and a 159-YPSPQ164 motif shared by mitochondria and proteobacteria. The phylogenetic analyses of diverse HSP70 sequences show monophyly of mitochondrial HSP70, including Cp-mtHSP70, within α-proteobacterial DnaK sequences. However, these analyses failed to resolve the relationships between lineages that are either deep branching or fast evolving, consistent with previous HSP70 analyses (25, 35). Importantly, the position of Cp-mtHSP70 within the mitochondrial clade was unambiguous, as was the position of the *C. parvum* cytosolic form. These data further confirm the presence of a mitochondrion in *C. parvum*.

As expected, the Cp-mtHSP70 presequence is rich in arginine (total of 3), alanine (total of 2), and serine (total of 7), while acid amino acids are absent. Like the iron sulfur cluster protein IscS (28), Cp-mtHSP70 has an R-2 motif that is preferred by the mitochondrial matrix processing peptidase (20, 36). This motif is not found in mitochondrial CpCpn60 or CplsCU (28, 40), but these observations are congruent with data showing that only about two-thirds of all targeting peptides have R in position –3 or –2 (20). Although the subcellular localization of Cp-mtHSP70 was predicted in silico to be mitochondrial, the algorithms for in silico predictions have been developed using sequences from organisms that are evolutionarily distant from protists (14). For example, TargetP was unable to identify a presequence in the *P. falciparum* mtHSP70 homologue even though it clusters unambiguously within mitochondrial HSP70 types. Therefore, in cases like these, in silico results must be viewed with caution.

Initially, we elucidated the in vivo ability of Cp-mtHSP70 presequence to target GFP to heterologous yeast mitochondria. When the Cp-mtHSP70 presequence was attached as an NH2 extension to a GFP reporter, this construct had the ability to direct the reporter to the mitochondrial network of yeast cells. Moreover, the targeting was as efficient as that for the yeast mtHSP70 homologue (43). Truncation of Cp-mtHSP70 at the extreme NH2 terminus indicates that residues beyond amino acid 20 of the presequence are essential for mitochondrial targeting, thus supporting the in silico prediction that these amino acids probably do form a bifacial amphiphilic α-helix crucial for mitochondrial import (1, 39). This is consistent with the observation that Δ1-10 constructs were 92% as efficient as the SSC1 control in targeting GFP to yeast mitochondria and that even Δ1-20 and Δ10-20 constructs targeted mitochondria with 79 and 75% efficiency, respectively.

Although yeasts are suitable for demonstrating mitochondrial targeting, as mentioned previously, most Apicomplexa (*E. tenella*, *P. falciparum*, and *T. gondii*) also have a chloroplast remnant—the apicoplast (17). Therefore, *T. gondii* was used as a surrogate host to test whether the Cp-mtHSP70 presequence would target the single mitochondrion, and not the apicoplast, of this apicomplexan, which contains both organelles. As expected, only the single mitochondrion of *T. gondii* was targeted by GFP when the *C. parvum* presequence was used. As mentioned previously, this is the first time *T. gondii* has been used as a surrogate for organelle targeting in *C. parvum*. Because there is no transient or stable transfection system for *C. parvum*, directly targeting the *C. parvum* relict mitochondrion is precluded.

Nevertheless, evidence for the presence of a relict mitochondrion was obtained using FITC-labeled anti-Cp-mtHSP70. Here, for the first time, immunofluorescence of the mitochondrion was observed both in *C. parvum* sporozoites and in intracellular stages. These immunofluorescence data indicate not only that a relict mitochondrion is present in sporozoites but that this organelle occurs in developmental stages that are multiplying within host cells. Previously, only immunogold labeling of transmission electron micrograph sections by anti-CpCpn60 was able to clearly delineate the relict mitochondrion.
Transmission electron micrographs indicate an intimate association of this organelle with the nuclear membrane and the rough ER which yields an appearance of a “ribosome-studded” mitochondrion (41). In many eukaryotes the outer nuclear membrane is continuous with the rough ER, contributing to both protein synthesis and secretion (37). Interestingly, the nuclear envelope of the apicomplexan *T. gondii* appears to be an intermediate compartment for secretory trafficking from the ER to the Golgi apparatus (23). It is likely that studies of protein trafficking in *C. parvum*, particularly the association of the relict mitochondrion with the rough ER, will also yield new insights into the unique biology and evolution of this apicomplexan.

As previously suggested, the HSP70 and Cpn60 mitochondrial-type chaperones in *C. parvum* probably serve as a part of the fundamental elements for the import and maturation of many proteins, including Fe-S clusters (28). Similarly, these mitochondrial chaperones have also been observed in modified mitochondria of other protists, i.e., hydrogenosomes of *Trichomonas vaginalis* and mitosome/cryptomon of *Entamoeba histolytica* and *Trachipleistophora hominis* (46, 50). Although recent experimental evidence suggests that the secondarily reduced mitochondrial compartment is essential for Fe-S cluster biosynthesis in *Giardia* (47), whether this is also a critical function of the *C. parvum* relict mitochondrion is still an open question (19, 28).

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