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Hsp60 Is Targeted to a Cryptic Mitochondrion-Derived Organelle ("Crypton") in the Microaerophilic Protozoan Parasite *Entamoeba histolytica*

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***Entamoeba histolytica* is a microaerophilic protozoan parasite in which neither mitochondria nor mitochondrion-derived organelles have been previously observed. Recently, a segment of an *E. histolytica* gene was identified that encoded a protein similar to the mitochondrial 60-kDa heat shock protein (Hsp60 or chaperonin 60), which refolds nuclear-encoded proteins after passage through organellar membranes. The possible function and localization of the amebic Hsp60 were explored here. Like Hsp60 of mitochondria, amebic Hsp60 RNA and protein were both strongly induced by incubating parasites at 42°C. 5′ and 3′ rapid amplifications of cDNA ends were used to obtain the entire *E. histolytica hsp60* coding region, which predicted a 536-amino-acid Hsp60. The *E. histolytica hsp60* gene protected from heat shock *Escherichia coli groEL* mutants, demonstrating the chaperonin function of the amebic Hsp60. The *E. histolytica* Hsp60, which lacked characteristic carboxy-terminal Gly-Met repeats, had a 21-amino-acid amino-terminal, organelle-targeting presequence that was cleaved in vivo. This presequence was necessary to target Hsp60 to one (and occasionally two or three) short, cylindrical organelle(s). In contrast, amebic alcohol dehydrogenase 1 and ferredoxin, which are bacteria-like enzymes, were diffusely distributed throughout the cytosol. We suggest that the Hsp60-associated, mitochondrion-derived organelle identified here be named "crypton," as its structure was previously hidden and its function is still cryptic.**

Entamoeba histolytica is a protozoan parasite which causes amebic dysentery and liver abscess in individuals in developing countries that cannot prevent its fecal-oral spread (34). Amebae are obligate fermenters which lack enzymes of oxidative phosphorylation, Krebs cycle enzymes, and pyruvate dehydrogenase (36). Indeed, amebae for a long time have been considered to be amitochondriate (28). Instead amebae have fermentation proteins (pyruvate:ferredoxin oxidoreductase [POR], ferredoxin, and alcohol dehydrogenases [ADH1, ADHE, and ADH3]) which are absent from most other eukaryotes and resemble those found in anaerobic bacteria (21, 25, 41, 55). Metronidazole, the best anti-amebic drug, is reduced and activated when it receives an electron from ferredoxin reduced by POR (22). Phylogenetic analyses of *E. histolytica* POR, ferredoxin, and ADHE strongly suggest that the genes encoding these fermentation enzymes derive from an anaerobic bacterium, although the route of entry of these genes into the cell and the identity of the ancestor are not clear (41, 47). Whether *E. histolytica* fermentation enzymes are cytosolic or are compartmentalized in organelles such as hydrogenosomes (described below) remains to be determined (30).

It is likely that *E. histolytica* also had a mitochondrial endosymbiont, as a putative amebic 60-kDa heat shock protein (Hsp60) aligns in phylogenetic trees with mitochondrial Hsp60 (7, 13, 15, 54). Hsp60 peptides, also known as chaperonin 60, form cylindrical structures within the mitochondrial matrix. In association with 10-kDa heat shock proteins (Hsp10), Hsp60 peptides are involved in the refolding of mitochondrial proteins after they have passed through two organellar membranes (16, 18, 42). Eubacterial GroEL proteins are homologous to Hsp60, while GroES proteins are homologues to Hsp10. Numerous *hsp60* genes have been identified from protozoan parasites, which have mitochondria (4, 37, 49, 50, 56). Further, mitochondrion-like Hsp60, Hsp10, and 70-kDa heat shock protein (Hsp70) are present in hydrogenosomes (anaerobic mitochondria) of the amitochondriate protozoan parasite *Trichomonas vaginalis*, which colonizes the vagina (5, 19, 30, 39). Like mitochondrial proteins, hydrogenosomal proteins, which also include POR, ferredoxin, and hydrogenase, are encoded by nuclear genes and contain organelle-targeting presequences at their amino termini (3, 8, 20, 42, 44, 45). Unlike mitochondria, hydrogenosomes lack circular DNAs containing rRNA and protein-encoding genes (30, 54).

A mitochondrion-like Hsp60, which is not induced by heat shock, is apparently located in the cytosol of *Giardia lamblia*, the amitochondriate protozoan parasite that colonizes the small intestine and causes diarrhea (40, 48). The presence of mitochondrial Hsp60 in early branching eukaryotes previously considered amitochondriate (*E. histolytica*, *G. lamblia*, and *T. vaginalis*) suggests that the common ancestor of all eukaryotes had the mitochondrial endosymbiont (5, 13, 15, 19, 26, 39, 40, 54). As well, genes encoding valyl-tRNA synthetase, which are believed to be of mitochondrial origin, have been cloned from *G. lamblia* and *T. vaginalis* (17). As amebae have no previously described organelles to which its Hsp60 might be targeted (28), it is not clear whether the amebic *hsp60* gene is expressed, where Hsp60 might be located, and what function the amebic Hsp60 might have.

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MATERIALS AND METHODS

Heat-induced expression of *E. histolytica hsp60* mRNA and Hsp60 protein. Axenically grown *E. histolytica* HM-1 in log phase was heat shocked by incubation for 1 h at 42°C. Heat-shocked and control amebae were lysed in guanidinium, and RNA was isolated by centrifugation through a cesium chloride cushion. Reverse transcription (RT)-PCR was performed with antisense (A1, ACTCCTCCCGTAAGTCTAGC) and sense (S1, GGAGATGGGACAACAA CAGC) primers specific for the amebic *hsp60* gene (7). As a positive control, RT-PCR was performed with antisense (CCGGTACCTTAGCAAGCATGAA TCTTAG) and sense (TAATACGACTCACTATAGGATCCATGAAAG) primers specific for the *amebapore A* gene (Fig. 1) (27).

Proteins from heat-shocked and control amebae were solubilized in lysis buffer (540 mg of urea/ml, 2% Triton X-100, 2% 2-mercaptoethanol, 2% ampholines [pH 3 to 10], 100 μg of E-64/ml) and electrophoresed on two-dimensional gels (31). Precast gels (Pharmacia Biotech AB, Uppsala, Sweden) contained ampholines from pH 3 to 10 in the first (isoelectric focusing) dimension and a gradient of acrylamide from 5 to 20% in the second (sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) dimension. Gels were stained with Coomassie blue, and a putative Hsp60 was identified as an ~56-kDa spot, which increased after heat shock. This Hsp60 spot was confirmed by running two-dimensional gels of transfected parasites overexpressing Hsp60 under an actin promoter, identifying the Hsp60 spot, excising it, and determining the amino-terminal sequence (see below).

5′ and 3′ RACE to determine the predicted amino and carboxy termini of the amebic *hsp60* gene. 5′ random amplification of cDNA ends (RACE) was performed with RNA from heat-shocked amebae to obtain the 5′ end of the *hsp60* coding region and a portion of the 5′ untranslated region (UTR) (11). Briefly, a first-strand cDNA was made with RT and the Hsp60-specific antisense primer A1 (described above). Terminal transferase and dCTP were used to add a poly(C) tail to the 3′ end of this *hsp60* cDNA. PCR was performed with this cDNA, a nested antisense primer (A2, GGAACTACACTTTGTGATGAGC) specific for the amebic *hsp60* gene, and a sense primer (S2, CCACGCGTCGA CTAGTACGGGGGGGGGGG) to poly(C) (Fig. 1). 3′ RACE was also performed with RNA from heat-shocked amebae. A first-strand cDNA was made with RT and an antisense primer (A3, CCACGCGTCGACTAGTACTTTTTT TTTTTTTTTT) to the poly(A) tail present on all amebic mRNAs. A first round of PCR was performed with this cDNA, antisense primer A3, and the Hsp60-specific sense primer S1 (described above). A second round of PCR was performed with A3 and a nested primer (S3, GCTGCAGTAAGAGCTCCAGG) specific for the amebic *hsp60* gene. The 5′ UTR of the *Hsp60* mRNA was 5-bp long (ATTTA), while the 3′ UTR was 23-bp long (ATTTTACTTTTAAAAAA AAAAAA), including a 12-bp poly(A) tail. The sequence of the predicted amebic Hsp60 was identical to that recently deposited in GenBank (accession no. AF02966) with the exception of a substitution of Thr for Ser at position 61 (7, 40). PCR of genomic DNA with primers from the start and stop codons of the *hsp60* gene (see below) produced the expected 1,611-bp product, demonstrating that introns are absent from the amebic *hsp60* gene. Like other amebic genes, 90% of the third codon positions in the *hsp60* coding region were A or T (9, 21, 25, 27, 33, 41, 55, 58). Protein sequences similar to the *E. histolytica* Hsp60 were obtained from GenBank by using BLAST (2).

Functional test of the amebic Hsp60 in the *Escherichia coli groEL* mutant. A novel gene (*hsp60-groEL*) was made encoding an *E. histolytica* Hsp60-*E. coli* GroEL fusion protein in which the amino and carboxy termini of the amebic Hsp60 were replaced by those of *E. coli* (Fig. 1) (18, 29). An *hsp60-groEL* gene was constructed by PCR. The sense primer (S4, A*CCATGG*CAGCTAAAGAC GTAAAATTCGGTAACGATTGTAGAGAAAATG) contained an *Nco*I site (italics) and encoded the first 10 amino acids of the bacterial GroEL protein (MAAKDVKFGN, underlined) and Asp^{11} to Asn^{15} of the amebic Hsp60 (double underlined). The antisense primer (A4, *GGATCC*TTACATCATGCCGCC CATGCCACCCATGCCGCCCATACCGCCAGCAGCGCCTAAGTCAGCT GCATCGTTTTTTGGTTCATCAGTTAT) contained a *Bam*HI site (italics) and encoded Ile^{526} to Pro^{530} of the amebic Hsp60 (underlined) and the carboxyl-terminal repeats of the *E. coli* GroEL protein (KNDAADLGAAGGMGGMG GMGGMM, double underlined). The pSE380-Hsp60-groEL construct was made by cloning the *Hsp60-groEL* gene into the pSE380 vector. This construct was transformed into an *E. coli groEL* mutant which was grown on plates containing tetracycline and ampicillin (18). Transformed and nontransformed (control) *E. coli groEL* mutants were streaked onto agar plates and incubated overnight at 37°C (permissive temperature at which *groEL* mutants and wild-type *E. coli* grow) and 44°C (the temperature at which *groEL* mutants die and wild-type *E. coli* grow).

Overexpression of native amebic Hsp60, epitope-tagged Hsp60, and epitope-tagged ferredoxin in transfected parasites. The coding region of the amebic *hsp60* gene was isolated from genomic DNA by PCR. The sense primer (S5, GC*GGTACC*ATGCTTTCATCTTCAAGTCAT) contained a *Kpn*I site (italics) and encoded the first six amino acids at the amino terminus of the parasite's Hsp60 protein (MLSSSSH, underlined). The antisense primer (A5, GC*GGAT CC*ATTAATTTCCTTTTTTATTGG) contained a *Bam*HI site (italics) and encoded six amino acids at the carboxyl terminus of the organism's Hsp60 protein (IKKEIN, underlined) (Fig. 1). The pJST4-Hsp60 plasmid was made by cloning the *hsp60* coding region into the *Kpn*I and *Bam*HI sites of the pJST4 amebic transformation vector, which contains the *neomycin phosphotransferase* gene and the gene to be overexpressed (here *hsp60*) under amebic *actin 1* gene promoters (Fig. 1) (12). The pJST4-Hsp60 plasmid was electroporated into *E. histolytica* HM-1 amebae, which weakly express the endogenous Hsp60 in axenic culture at 37°C, and selected with G418 to a final concentration of 50 μg/ml.

The location of the Hsp60 protein was determined on two-dimensional protein gels by identifying the ~56-kDa, Coomassie blue-stained spot, the expression of

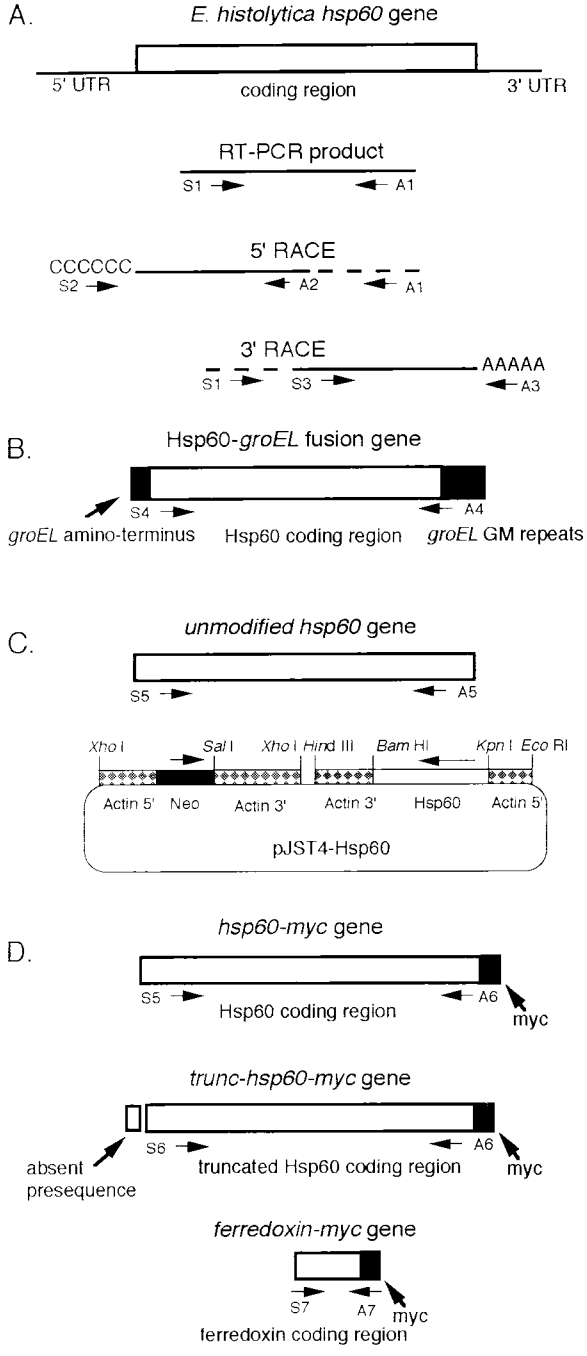

FIG. 1. Cartoon of RT-PCR products and constructs for testing function and localization of amebic Hsp60. (A) Amebic *hsp60* RT-PCR products. Primers are defined in Materials and Methods. (B) Construct used to test amebic Hsp60 in *groEL* bacteria. GroEL protein sequences are in black. (C) pJST4-Hst60 plasmid used for stable transfection of amebae. The coding regions of the amebic *hsp60* gene (striped box) and bacterial neomycin phosphotransferase (black box) were expressed under amebic *actin 1* gene promoters. (D) *hsp60-myc*, *trunc-hsp60-myc*, and *ferredoxin-myc* constructs.

which was increased in transfected but not nontransfected parasites. This was the same spot that was increased in heat-shocked parasites (see above). In addition, two-dimensional gels were transferred to polyvinylidene difluoride filters and stained with Ponceau; the Hsp60 spot was excised, and its amino-terminal sequence was determined by William Lane at the microchemistry facility in the biological laboratories of Harvard University.

A novel gene (*hsp60-myc tag*) encoding the amebic Hsp60 labeled with a myc epitope at its carboxy terminus was constructed by PCR and the sense primer S5 (described above). The antisense primer (A6, GC*GGATCC*TTATAAATCTTC TTCTGAAATTAATTTTTGTTCATTAATTTCCTTTTTTATTGG) contained a *Bam*HI site (italics) and encoded six amino acids at the carboxyl terminus of the organism's Hsp60 protein (IKKEIN, underlined) and a myc epitope (EQKLISEEDL, double underlined) (Fig. 1) (10). The *hsp60-myc* tag gene was cloned into the pJST4 amebic transformation vector to make a construct called pJST4-Hsp60-Myc, which was electroporated into *E. histolytica* HM-1 amebae. A second novel gene (*trunc-Hsp60-myc tag*), encoding a myc-tagged Hsp60 that was missing the amino-terminal presequence, was constructed with the antisense primer A6 and a sense primer (S6, GA*GGTACC*ATGTTATCAGGAATAA AG) containing a *Kpn*I site (italics) and encoding Met and Hsp60 beginning at Leu^{15} (underlined). This construct was cloned into the pJST4 vector and transfected into amebae as described above.

To localize ferredoxin, amebae were transfected with pJST4 containing a novel gene (*ferredoxin-myc*) encoding the amebic ferredoxin labeled with a myc epitope at its carboxy terminus (Fig. 1). The coding region of the amebic *ferredoxin* gene was isolated from genomic DNA by PCR. The sense primer (S7, GC*GGTACC* ATGGGAAAGATCACTATTGTT) contained a *Kpn*I site (italics) and encoded the first seven amino acids at the amino terminus of the parasite's ferredoxin (MGKITIV, underlined) (21). The antisense primer (A7, GCGGATCCTTATA AATCTTCTTCTGAAATTAATTTTTGTTCAACTCCTTG) contained a *Bam*HI site and encoded three amino acids at the carboxyl terminus of the organism's ferredoxin (QGV, underlined) and the myc epitope (EQKLISEEDL, double underlined) (Fig. 1).

Fluorescence confocal microscopy. For indirect immunofluorescence and confocal microscopy, amebic trophozoites were fixed with 2% paraformaldehyde for 10 min at 4°C and permeabilized by incubation with 1% Nonidet P-40. To visualize Hsp60 in nontransfected parasites, a monospecific polyclonal rabbit antibody to amebic Hsp60 was made by immunizing animals with a multiantigenic peptide (MAP) to 23 amino acids (SVGSLIATSEALITDEPIKKEIN) at the carboxy terminus of the amebic Hsp60. This rabbit anti-amebic Hsp60 antibody was purified on a column composed of the amebic Hsp60 MAP. This antibody was incubated with fixed and permeabilized amebae for 60 min at 37°C in phosphate-buffered saline and 2% bovine serum albumin (PBS-BSA). Parasites were washed four times and immunodecorated for 60 min at 37°C with fluorescein-labeled goat anti-rabbit antisera.

Amebae transfected with plasmids encoding Hsp60 or ferredoxin with myc-epitope tags were immunostained with a monoclonal anti-myc antibody (10 μg/ml) in PBS-BSA (10). Organisms were washed four times and immunodecorated for 60 min at 37°C with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse sera. Negative controls include nontransfected parasites and nonimmune mouse sera.

To visualize amebic alcohol dehydrogenase 1, amebae were fixed and immunostained with a polyclonal rabbit anti-alcohol dehydrogenase 1 antibody made to a glutathione *S*-transferase fusion protein and immunodecorated with a fluorescein-labeled goat anti-rabbit antisera (25, 46). Fluorescently labeled parasites were observed with a Leica NT-TCS confocal microscope fitted with argon and krypton lasers. Images of amebae were recorded in 512 image size format with a 40× Planapo objective.

To identify pinocytotic vesicles, amebae were incubated with 1-mg/ml FITC-dextran for 30 min at 37°C, washed four times in PBS, and fixed with paraformaldehyde. To determine whether the amebae contained an organelle with an electrochemical gradient, parasites were incubated with 10 μg of rhodamine 123/ml or 10 μg of JC-1 dye/ml and observed with the fluorescence microscope (6, 35). As a positive control, *Leishmania enriettii* parasites, which contain a mitochondrion with a strong electrochemical gradient, were incubated with both dyes. To look for extranuclear DNA, parasites were fixed with 1% formaldehyde to prevent pinocytosis, stained with 1-μg/ml Hoechst dye 33258 for 30 min at 37°C, and examined with the fluorescence microscope.

RESULTS AND DISCUSSION

Heat-shock-induced expression of amebic Hsp60. Amebic hsp60 mRNAs, detected by RT-PCR, were weakly present in control amebae incubated at 37°C and were increased in parasites incubated at 42°C (Fig. 2). In contrast, RT-PCR products of mRNAs for amebapore A, which is a lysosomal protein involved in killing bacteria and/or host cells, remained constant during the temperature shift from 37 to 42°C. By similar methods, we determined that amebic mRNAs encoding homologues of Hsp70 (also known as BiP), Hsc70, and inositol

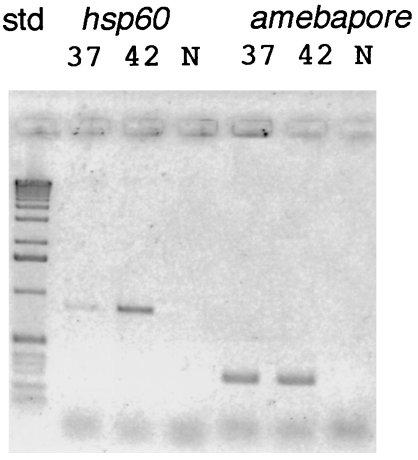

FIG. 2. Results of RT-PCR with primers to the amebic Hsp60 by using total RNA from amebae cultured in the absence of heat shock (37°C) and after heat shock (1 h at 42°C). An ethidium-stained agarose gel of RT-PCR products was electronically reversed for ease of reproduction. Control RT-PCR with primers to the amebapore was performed with the same RNA targets. Lanes 3 and 6 contain negative controls (N) with no target RNA. These RT-PCR results are qualitative rather than quantitative.

kinase were also increased with heat shock (unpublished data) (33). Two-dimensional protein gels of heat-shocked parasites versus controls without heat shock showed increased expression of a 56-kDa protein, which coincided with that of amebae transfected with their own *hsp60* gene under an actin gene promoter (Fig. 3). These results, which are qualitative rather than quantitative, demonstrate that the amebic Hsp60 is appropriately named and may serve functions in the parasite comparable to those described for other eukaryotic Hsp60 proteins (16, 18, 42).

Chaperonin function of the amebic Hsp60 in an *E. coli groEL* mutant. The entire 1,608-bp amebic *hsp60* coding region, encoding a putative 536-amino-acid (M_r, 56,788) Hsp60, was obtained by 5′ and 3′ RACE, with RNA from heat-shocked parasites (Fig. 1) (11). To test the function of the amebic Hsp60, the *E. histolytica hsp60* gene was expressed in

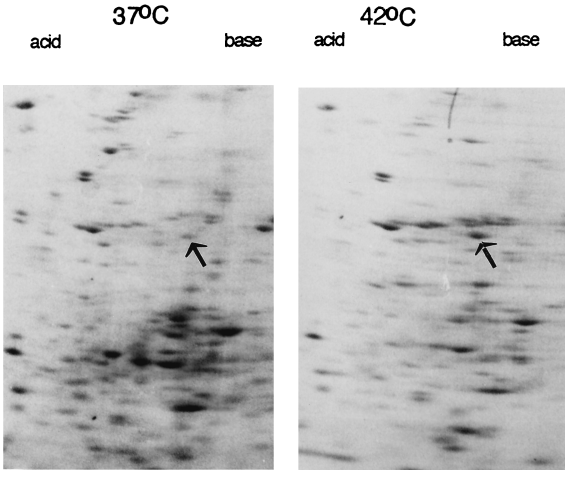

FIG. 3. High-power view of two-dimensional protein gels of amebae incubated at 37°C and after heat shock (42°C). Arrows mark putative Hsp60s, which were in the same location as recombinant Hsp60 expressed in amebae transfected with the pJST4-Hsp60 plasmid (data not shown). Note that the putative Hsp60 is not the only protein which changes in abundance with heat shock.

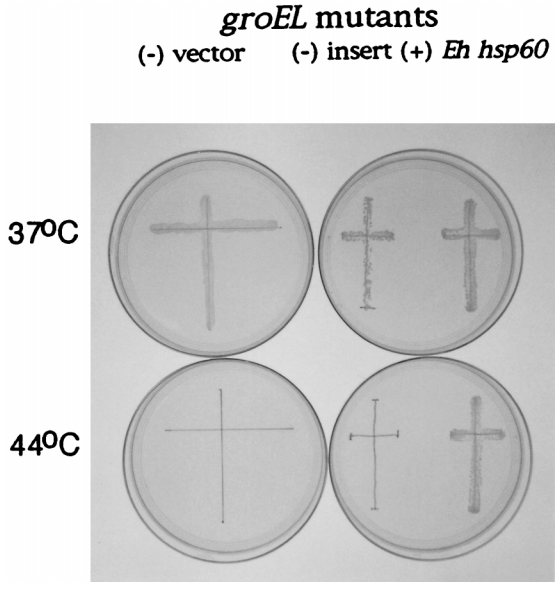

FIG. 4. Plate showing *groEL* mutants incubated at 37°C (permissive temperature) or 44°C (nonpermissive temperature). Each *groEL* mutant was streaked as a cross, which was marked with a pen. A nontransformed *groEL* mutant was maintained on plates containing tetracycline (left), while *groEL* mutants transformed with the pSE380 vectors were maintained on plates containing tetracycline and ampicillin (right). A *groEL* mutant complemented with the amebic Hsp60-*E. coli groEL* fusion gene (*Eh hsp60*) grew at both temperatures. In contrast, the control *groEL* mutant [(−) vector] failed to grow at 44°C. Similarly, a *groEL* mutant which was transformed with the pSE380 vector without an insert [(−) insert] failed to grow at 44°C.

an *E. coli groEL* mutant. This mutant lacks an Hsp60-like chaperonin and so is killed when incubated at 44°C (18). To increase the likelihood of success, the *E. histolytica hsp60* gene was modified at its 5′ and 3′ ends to encode an amebic Hsp60 protein with amino and carboxy termini like those of the *E. coli* GroEL protein. The *groEL* mutant transfected with a plasmid containing the modified *E. histolytica hsp60* gene survived incubation at 44°C (Fig. 4). Control *groEL* mutants, which were not transfected or were transfected with a plasmid containing no foreign gene, died at 44°C. These results demonstrate that the amebic Hsp60 can act as a chaperonin in *E. coli* and suggest a similar function for Hsp60 in the parasite. It remains to be determined whether a wild-type amebic *hsp60* gene, rather than the *hsp60-groEL* fusion gene used here, is able to complement the *groEL* mutant (29).

Absence of Gly and Met repeats at the carboxy terminus of the predicted amebic Hsp60. The carboxyl terminus of the *E. histolytica* Hsp60 lacked Gly and Met residues, which are repeated at the carboxy termini of all other Hsp60 and GroEL proteins except that of *Leishmania major* (Fig. 5) (2, 4, 5, 18, 19, 23, 37, 39, 45, 49–51, 56, 57). The absence of the carboxy terminal repeats and the somewhat short amebic amino terminus of the *E. histolytica* Hsp60 (see below) made it 20 to 53 amino acids shorter than other eukaryotic Hsp60 proteins. The functional significance of the absence of carboxy-terminal repeats of the amebic Hsp60 is unclear. A truncated *E. coli* GroEL protein lacking the Gly and Met repeats behaves like the wild-type GroEL protein in vitro but has numerous subtle deficiencies compared with the wild-type GroEL protein in vivo (29).

Hydrogenosome-like, organelle-targeting presequence at the amino terminus of the *E. histolytica* Hsp60. The amino portion of the amebic Hsp60, which includes 55 amino acids not previously identified, aligned without gaps with other Hsp60 or GroEL proteins beginning at Lys^{11} (Fig. 5) (2, 4, 5, 16, 18, 19, 23, 37, 39, 44, 45, 49–51, 56, 57). However, the amebic Hsp60 sequence contained numerous unique amino acids from Lys^{12} to Val^{29} and from Thr^{44} to Val^{56}, further demonstrating its difference from other mitochondrial Hsp60s.

Eukaryotic Hsp60s have mitochondrion-targeting presequences at their amino-termini, while bacterial GroEL proteins, which are cytosolic, lack such presequences (Fig. 5) (8, 18, 42, 44, 51). Mitochondrion-targeting presequences, which are proven for the Hsp60s of humans, *Leishmania major*, and *Cucurbita* sp. (pumpkin) and are putative for the rest of the Hsp60, are 20 to 30 amino acids long, are enriched in Ser and Arg, lack negatively charged amino acids, and contain an endopeptidase cleavage site. The amebic Hsp60 contained an amino-terminal decapeptide (Met-Leu-Ser-Ser-Ser-Ser-His-Tyr-Asn-Gly), which was distinct from those of other eukaryotic Hsp60s but included multiple amino acids (underlined) present at the amino-terminus of most *T. vaginalis* proteins targeted to hydrogenosomes (Fig. 6) (3, 20). Similarly, the amino terminus of the amebic nicotinamide nucleotide transhydrogenase (NNT) (MSTSSSIEEEVFNYMKITNNFVSVG NIIIS), a homologue of the mitochondrial NNT, contains numerous Ser residues (7, 58). In vitro experiments with *T. vaginalis* ferredoxin demonstrated the importance of Leu^2 (also present in the amebic Hsp60) for targeting ferredoxin to hydrogenosomes (3).

The amebic amino-terminal Hsp60 decapeptide is apparently part of an organelle-targeting presequence, because it and 11 other amino acids were removed from Hsp60 in vivo. The amebic Hsp60, which was overexpressed in cultured trophozoites and isolated on two-dimensional gels similar to that shown in Fig. 3, had an amino terminus that began at Asn^{22} (Fig. 6). The 21-amino-acid amebic Hsp60 presequence is similar in length to mitochondrial presequences and somewhat longer than hydrogenosomal presequences. The amebic Hsp60 presequence has an Arg residue at −2 relative to the cleavage site, as is the case for most mitochondrial and hydrogenosomal presequences (Fig. 5 and 6) (3, 8, 20, 42, 44). These results suggest that the organelles to which amebic Hsp60 is targeted (see below) have receptors and endopeptidases which are similar to those of mitochondria and hydrogenosomes (42).

Organellar location of the amebic Hsp60 and cytosolic location of alcohol dehydrogenase 1 and ferredoxin. Amebic Hsp60 was localized to a short cylindrical organelle in nontransfected parasites by using antibodies to a peptide at the carboxy terminus of amebic Hsp60 (Fig. 7A). Although many cells contained one Hsp60-associated organelle, some cells contained two or three organelles. Nearly identical results were obtained with transfected parasites overexpressing Hsp60 with a carboxy-terminal myc tag by using anti-myc antibodies (Fig. 1 and 7B) (10). A truncated myc-tagged Hsp60, which lacked 13 amino acids at the amino terminus, went to the cytosol of transfected parasites rather than to the organelle (Fig. 7C). This result demonstrates that targeting of Hsp60 to the organelle is dependent upon the presence of multiple Ser and/or positively charged residues at its amino terminus, as has been shown for hydrogenosomal ferredoxin or mitochondrial proteins (3, 8, 42).

Absent from the Hsp60-containing organelles was the fermentation enzyme alcohol dehydrogenase 1, which was located in the parasite cytosol by polyclonal rabbit antibodies (Fig. 7D) (25). Also absent from the Hsp60-containing organelles was ferredoxin, which was overexpressed with a myc tag in transfected parasites and detected with anti-myc antibodies (Fig. 7E) (10, 21). Hsp60-associated organelles were distinct from

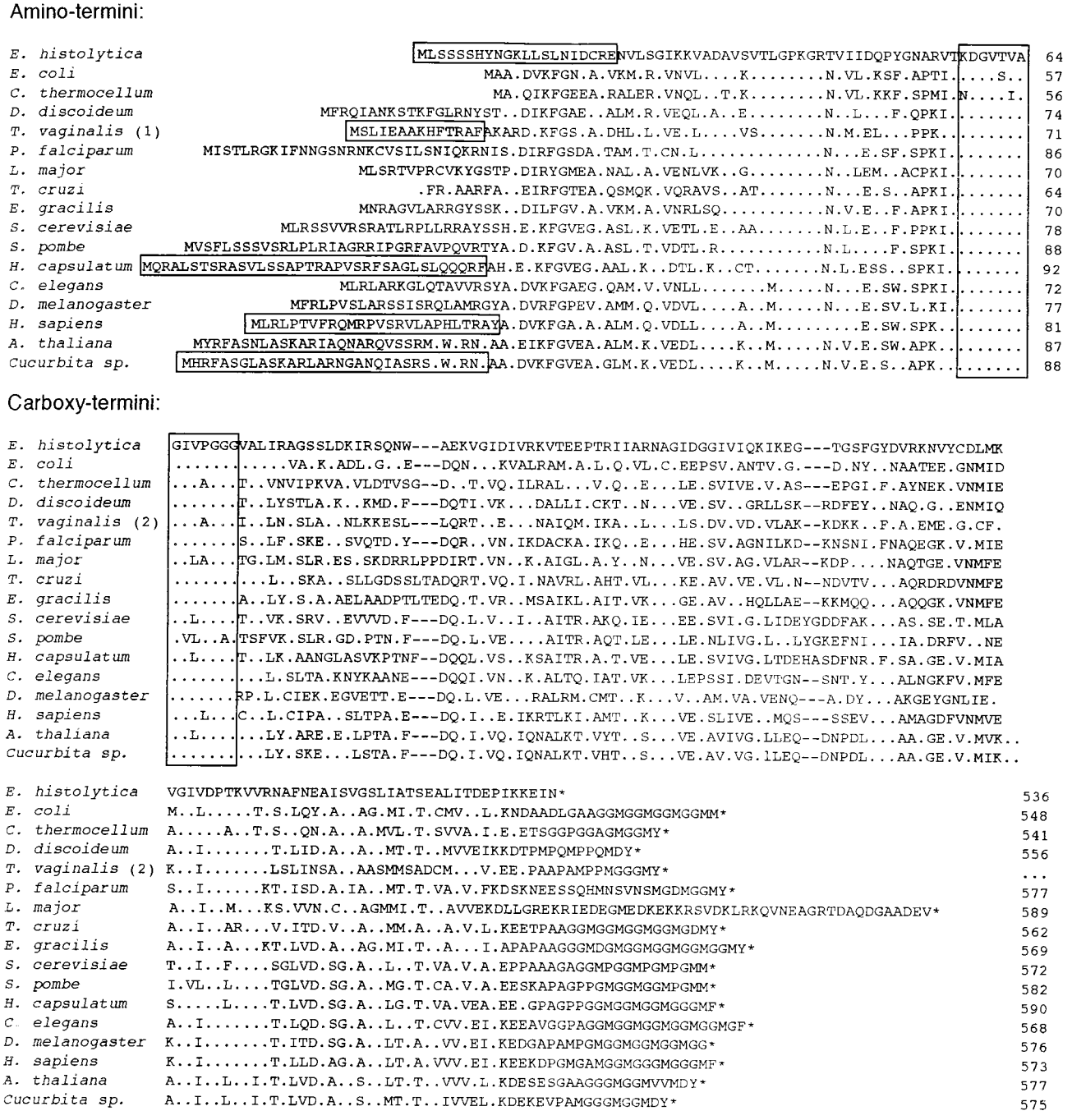

FIG. 5. Alignment of the amino and carboxyl termini of the predicted *E. histolytica* Hsp60 in single-letter code with GroEL proteins of *E. coli* (X07850) (18) and *Clostridium thermocellum* (Z68137) and Hsp60s of *Dictyostalium discoideum* (U72247), *T. vaginalis* (1, U26966 [5] and 2, U57000 [39]), *Plasmodium falciparum* (U38963) (50), *L. major* (U59320) (37), *Trypanosoma cruzi* (X67473) (49), *Euglena gracilis* (U49053) (56), *Saccharomyces cerevisiae* (M33301) (23), *Schizosaccharomyces pombe* (D50609) (57), *Histoplasma capsulatum* (L11390), *Caenorhabditis elegans* (L36035), *Drosophila melanogaster* (X99341), *Homo sapiens* (M22382) (45), *Arabidopsis thaliana* (Z11547), and *Cucurbita* sp. (pumpkin, X70868) (51). Periods indicate amino acids identical to those of the amebic Hsp60, dashes indicate gaps, and asterisks indicate stop codons. Vertical boxes indicate location of primers used previously to identify a segment of the amebic Hsp60 (7). Horizontal boxes indicate proven organelle-targeting presequences of amebic, trichomonad, *Histoplasma*, human, and pumpkin Hsp60. The *T. vaginalis* Hsp60 sequence is a composite of two published sequences, each of which is incomplete, so the total length could not be determined.

spherical vacuoles containing pinocytosed dextran (Fig. 7F). Organelles containing Hsp60 were also distinct from spherical secretory vesicles where the amebic chitinase, which has a secretory signal sequence at its amino terminus that obeys the -3, -1 rule, goes when expressed in transfected parasites under an actin promoter (data not shown) (9, 12, 53).

Consistent with their cytosolic localization, amebic ADH1 and ferredoxin lack either signal sequences or organelle-tar-

E. histolytica Hsp60	MLSSSSHYNGKLLSLNIDCRE
T. vaginalis Hsp60	MSLIEAAKHFTRAF
" α-succinyl-CoA synthetase 2	MLSSFERN
" β-succinyl-CoA synthetase 1	MLSASFNFERN
" malic enzyme 1	MLTASVSVPVRN
" adenylate kinase	MLSGVSRN
" ferredoxin	MLSQVCRF
" POR	MLRNF

FIG. 6. Alignment of the amino-terminal organelle-targeting presequence of *E. histolytica* Hsp60 with those of *T. vaginalis* hydrogenosomal proteins (3, 20). Dashes indicate amino acids identical to those of the amebic Hsp60. To identify the amebic presequence, an Hsp60 spot was excised from a two-dimensional gel of amebic proteins and sequenced by Edman degradation.

geting presequences (8, 21, 25, 53). Amebic POR and ADHE, which also lack signal sequences or organelle-targeting presequences, have been found on the surface of amebae and in one report in organelles (POR) (8, 38, 41, 43, 55). How amebic POR and ADHE get to these locations is not clear. In contrast, hydrogenosomal proteins of *T. vaginalis*, which include POR, ferredoxin, and succinate dehydrogenases, have organellar-targeting presequences (Fig. 6) (3, 20). Finally, a putative *G. lamblia* Hsp60 has been localized with heterologous antibodies to the parasite cytosol (39, 48).

Apparent lack of DNA or electrochemical gradient in the Hsp60-associated organelle. In order to further characterize the Hsp60-associated organelle, amebae were incubated with rhodamine 123 or JC-1, which are cationic and hydrophobic dyes that target organelles with a strong electrochemical gradient (6, 35). Although mitochondria of *L. enriettii* were strongly labeled with each dye, neither dye labeled any structures within amebae. These results suggest that there is no strong electrochemical gradient within the Hsp60-associated organelles of amebae. A weak electrochemical gradient within the organelle may be made with ATP produced in the cytosol by substrate-level phosphorylation (36). Further, the Hsp60-associated organelles failed to stain with Hoechst dye, which stained the kinetoplastid of *L. enriettii*. Although the possibility of a small genome remaining within the Hsp60-associated organelle cannot be ruled out, these results suggest that DNA from these organelles has shifted to the nucleus, as has been shown for hydrogenosomes of trichomonads (30). We were unable to confirm recent observations of large cytoplasmic accumulations of DNA within cultured amebae (32).

Likelihood that the *E. histolytica* Hsp60 functions as an organellar chaperonin. Strong circumstantial evidence suggests that the *E. histolytica* Hsp60 functions as a chaperonin within a mitochondrion-derived organelle. First, Hsp60 is expressed after heat shock. Second, the amebic *hsp60* gene complements an *E. coli groEL* mutant. Third, the primary structure of amebic Hsp60 is similar to those of other Hsp60 and GroEL proteins (7, 39). Fourth, an amino-terminal presequence is

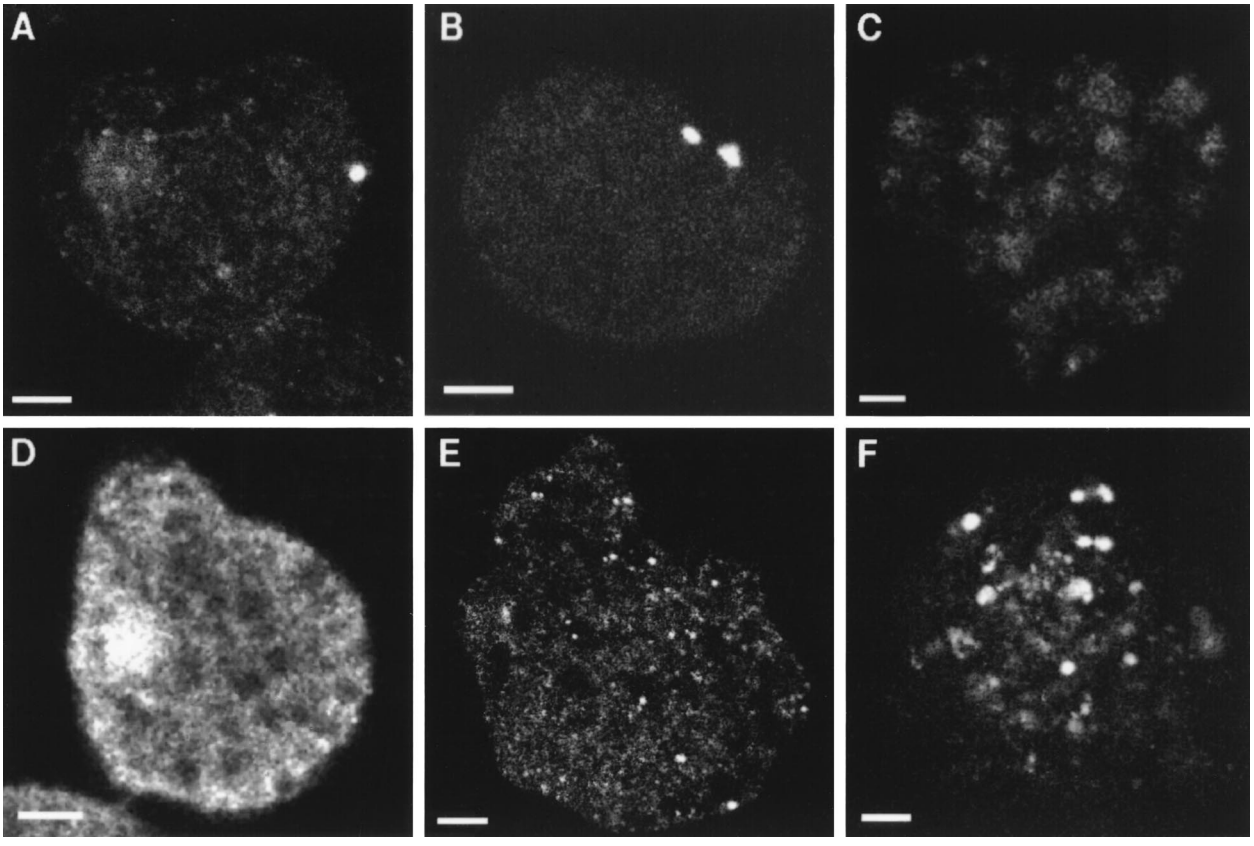

FIG. 7. Fluorescence confocal micrographs of *E. histolytica* trophozoites localizing an Hsp60-associated organelle and contrasting it with the amebic cytosol or pinosomes. Antibodies to Hsp60 (A) identified a short, cylindrical organelle in a nontransfected parasite. Antibodies to a myc identified two similar organelles in transfected amebae overexpressing Hsp60 with a C-terminal myc tag (B). However, most parasites labeled with anti-Hsp60 or anti-myc antibodies contained a single organelle. In contrast, anti-myc antibodies had a cytosolic distribution when parasites were transfected with a truncated *hsp60* gene encoding an Hsp60 lacking Ser and positively charged residues in its presequence (C). Antibodies to ADH1 in nontransfected parasites (D) gave a cytosolic distribution, with some labeling of the nucleus. Similarly, anti-myc antibodies had a cytosolic, if somewhat granular, distribution when parasites were transfected with a ferredoxin gene (E) encoding a peptide with a myc tag at its carboxy terminus. Pinocytosed FITC-dextran by nontransformed amebae fills hundreds of vesicles (F), most of which were small.

cleaved from amebic Hsp60 in vivo and Hsp60 is located within an organelle. In addition, *E. histolytica* contains genes encoding proteins homologous to cytosolic Hsp70 and 14-3-3 (also known as mitochondrial stimulation factor or MSF) (1, 12a, 33). Cytosolic Hsp70s maintain proteins targeted to the mitochondria in a translocation-competent conformation, while MSFs recognize mitochondrial import signals on mitochondrial precursor proteins and target them to receptors on mitochondria (1, 42).

Unknown function of the amebic mitochondrion-derived organelle, tentatively named here "crypton." The Hsp60-associated organelles identified here are small and rare (often one per cell), so they may have been overlooked in electron microscopic studies of amebae (28). These organelles may have been seen with anti-POR antibodies, although our results with ferredoxin are contradictory (38). They may also have been seen by H. N. Ray and coworkers with histological stains in the pre-electron microscopy era (reference 7 and references therein). The amebic organelle is unique among mitochondrion-derived organelles because it contains neither enzymes of oxidative phosphorylation (like mitochondria) nor fermentation enzymes (like hydrogenosomes of *T. vaginalis*) (3, 5, 19, 20, 30, 39).

The paucity of the amebic Hsp60-associated organelles and their small size are reminiscent of petite mitochondria of anaerobically grown yeast or the atrophic mitochondria of bloodstream trypanosomes, which use glycolysis rather than oxidative phosphorylation (14, 52). The difference between the amebic organelles and the mitochondria of petite yeast or bloodstream trypanosomes is that the latter change back to operative mitochondria when yeast are exposed to oxygen or trypanosomes are transferred to the insect vector. We suggest that the amebic Hsp60-associated, mitochondrion-derived organelle identified here be named crypton, as its structure was previously hidden and its function is still cryptic. Recently, apicomplexa have been shown to have a plastid organelle bound by four membranes, the function of which is not yet certain (24).

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