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# A Novel ADP/ATP Transporter in the Mitosome of the Microaerophilic Human Parasite *Entamoeba histolytica*

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## Summary

**Recent data suggest that microaerophilic and parasitic protozoa, which lack oxidative phosphorylation, nevertheless contain mitochondrial homologs [1–6], organelles that share common ancestry with mitochondria. Such widespread retention suggests there may be a common function for mitochondrial homologs that makes them essential for eukaryotic cells. We determined the mitochondrial carrier family (MCF) complement of the *Entamoeba histolytica* mitochondrial homolog, also known as a crypton [5] or more commonly as a mitosome [3]. MCF proteins support mitochondrial metabolic energy generation, DNA replication, and amino-acid metabolism by linking biochemical pathways in the mitochondrial matrix with those in the cytosol [7]. MCF diversity thus closely mirrors important facets of mitochondrial metabolic diversity. The *Entamoeba histolytica* mitosome has lost all but a single type of MCF protein, which transports ATP and ADP via a novel mechanism that is not reliant on a membrane potential. Phylogenetic analyses confirm that the *Entamoeba* ADP/ATP carrier is distinct from archetypal mitochondrial ADP/ATP carriers, an observation that is supported by its different substrate and inhibitor specificity. Because many functions of yeast and human mitochondria rely on solutes transported by specialized members of this family, the *Entamoeba* mitosome must contain only a small subset of these processes requiring adenine nucleotide exchange.**

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## Results and Discussion

### Identification of an MCF Homolog on the *Entamoeba histolytica* Genome

We identified a homolog of the mitochondrial carrier family (MCF) on the *Entamoeba histolytica* genome (TIGR) with BLAST searches. The translated protein contains a tripartite structure [8], signature motifs (IPR001993), and other features typical of MCF members (Figure 1A). The *Entamoeba* carrier contains 276 amino acid residues and is therefore the smallest MCF member so far identified, and the loop regions linking the transmembrane $\alpha$-helices are extremely short [9] (Figure 1B). Most eukaryotes have many mitochondrial carriers, typically between 30 and 60, that transport different substrates required or produced by the mitochondrion [7]. In contrast, the genomes of the malaria parasite *Plasmodium falciparum* [10] and the intestinal parasite *Cryptosporidum parvum* [11] have only nine and five mitochondrial carriers, respectively. This may reflect the propensity of parasites to use host metabolites rather than to make their own, leading to the elimination of metabolic pathways and transport steps surplus to requirements. The reductionist tendency has been taken much further by *Entamoeba histolytica* because we found only a single mitochondrial carrier on its genome.

### Localization of the *Entamoeba* MCF Protein

The *Entamoeba* mitosome imports chaperonin 60 (Cpn60) [3, 5], a protein that is of $\alpha$-proteobacterial ancestry and is typically found in mitochondria, where it is involved in the ATP-dependent folding of organellar proteins [12]. The *Entamoeba* Cpn60 clusters with the *Dictyostelium* mitochondrial protein in phylogenetic analyses [13], and it has an amino-acid extension similar to known mitochondrial targeting signals [3, 14]. Deletion of this extension prevents import of Cpn60 into the mitosome, but import can be restored by addition of a functional mitochondrial-targeting signal from *Trypanosoma cruzi* [3].

Western blotting (Figure 2A) showed that the *Entamoeba* MCF protein occurred in the same *Entamoeba* cell fractions as those containing Cpn60, strongly suggesting that it is in the same compartment. Mitochondrial carriers are targeted to, and inserted into, the mitochondrial inner membrane via a second import pathway that does not require an N-terminal leader se-

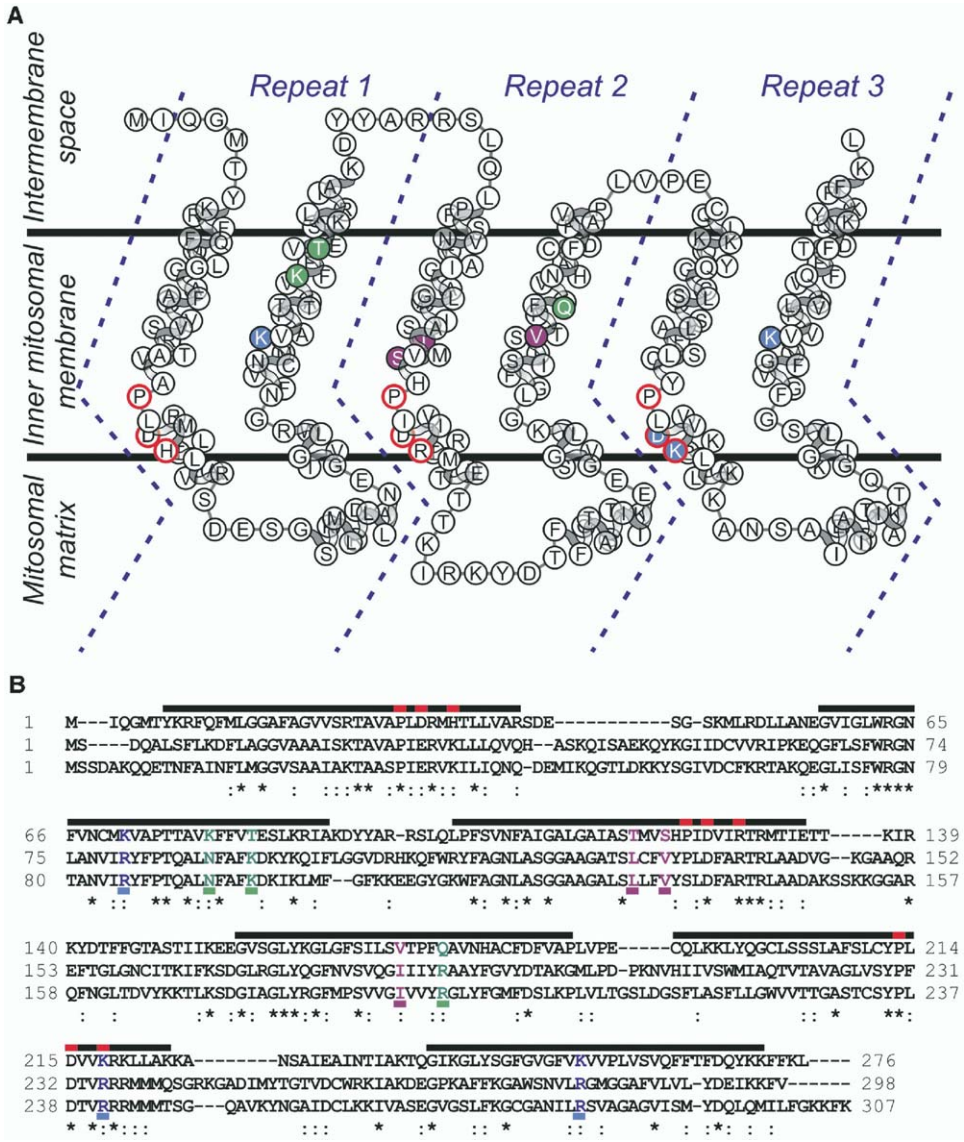


Figure 1. Sequence Analysis of the Adenine Nucleotide Carrier from *Entamoeba histolytica*

(A) The putative membrane topology of the carrier; the schematic representation indicates the key residues of the signature motif (red circles) and the three amino-acid sequence repeats (bordered by blue dashed lines) that are typical for members of the mitochondrial carrier family. Colored circles represent the amino-acid residues of the *Entamoeba* carrier; these residues correspond to the bovine ADP/ATP carrier residues that are involved in binding of carboxy-atractyloside. Shown in green are amino-acid residues that are involved in the binding of the sulfate groups of carboxy-atractyloside; in blue, residues that bind the carboxy group of the inhibitor; and in purple, residues that have van der Waals interactions [9].

(B) Alignment of the amino sequence of the *Entamoeba* adenine nucleotide carrier (top), the bovine ADP/ATP carrier (middle), and the yeast ADP/ATP carrier 3 (AAC3) (bottom). The black bars indicate the position of the transmembrane regions in the amino-acid sequence as deduced from the structure of the bovine ADP/ATP carrier [9]. The red bars indicate key amino-acid residues of the signature motif. The blue, green, and purple bars indicate the position of the key residues involved in the binding of carboxy-atractyloside by the bovine ADP/ATP carrier as in (A).

quence [15]. The *Entamoeba* carrier was expressed in yeast and correctly targeted to the inner membrane of mitochondria (Figures 2B–2D). The possession of an MCF protein, complete with appropriate signals to guide heterologous mitochondrial import [16], provides additional strong evidence that the *Entamoeba* mitosome is a mitochondrial homolog [3, 5].

## Functional Characterization of the *Entamoeba* MCF Protein

The *Entamoeba* carrier was expressed in the bacterium *Lactococcus lactis* [17]. Membrane vesicles containing the *Entamoeba* carrier took up radio-labeled ATP in exchange for ADP (see the Supplemental Data available with this article online). The uptake of radio-labeled

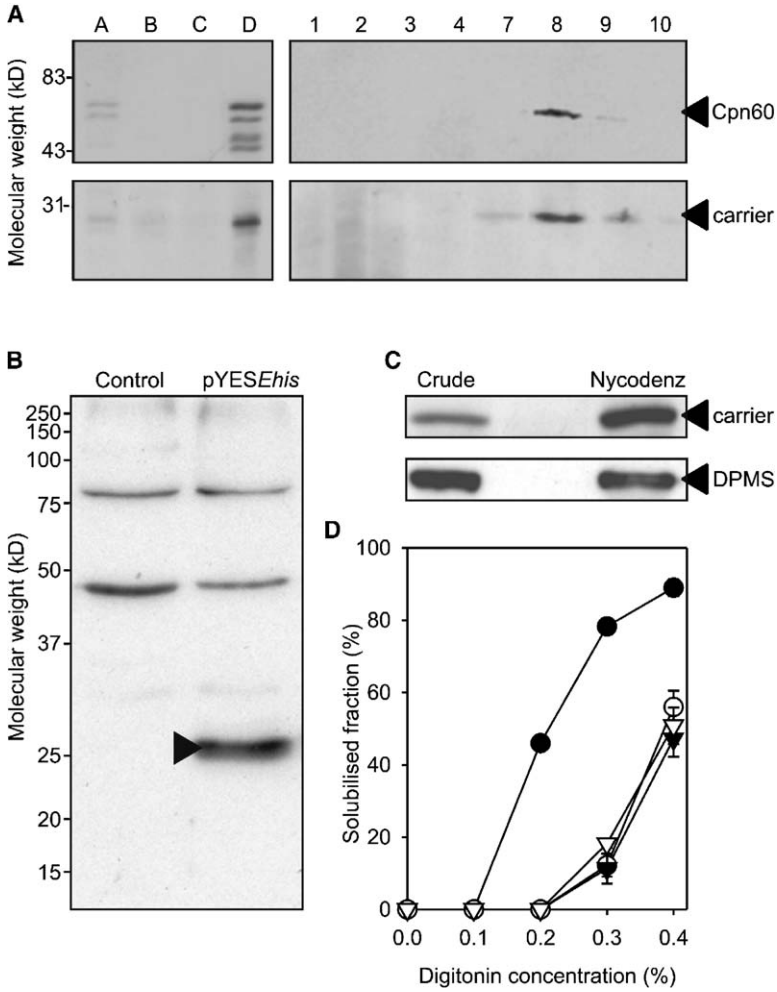


Figure 2. Cellular Distribution of the Carrier in *Entamoeba histolytica* and Targeting of the Mitosomal Carrier to Yeast Mitochondria

(A) Representative Western blots showing the subcellular distribution of Cpn60 (top) and of the adenine nucleotide carrier (bottom) in *E. histolytica*. Crude extracts were fractionated by differential centrifugation: (A) crude extract; (B) nuclear fraction; (C) high-speed supernatant (cytosolic fraction); and (D) high-speed sediment. The presence of multiple bands reacting to the Cpn60 antibody in lane D is caused by protein degradation—the top band corresponds to full-length Cpn60 [3]. The high-speed sediment was further fractionated on Percoll density gradients; see the Supplemental Data for details.

(B) Western blot of isolated yeast mitochondria of the control strain and strain that contained the expression vector with the gene coding for the *Entamoeba* carrier. The carrier was detected with antibodies against a synthetic peptide, corresponding to the region 84–97. The molecular weight of the mitosomal carrier is indicated by the triangle.

(C) The crude mitochondrial preparation contained a contamination with ER/Golgi, as could be detected by antibodies against dolichol phosphate mannose synthase (DPMS), but no detectable levels of nuclei or peroxisomes. The crude preparation was further purified by a Nycodenz gradient [32], and the *Entamoeba* carrier was enriched four times, and the ER/Golgi contamination decreased by 30%, showing that the carrier was not associated with the ER/Golgi impurities.

(D) Purified intact mitochondria were subjected to differential solubilization with digitonin. The mitosomal carrier (open triangles) solubilized at the same concentration as the endogenous mitochondrial cytochrome c-oxidase (open circles) and the ADP/ATP carrier 2 (closed triangles) from the inner mitochondrial membrane, whereas the porin from the outer membrane (closed circles) solubilized at a much lower detergent concentration. The values are the mean of three quantifications.

ADP in exchange for ADP was prevented by the addition of excess ATP, ADP, and AMP and to a lesser extent by phosphate, cAMP, dATP, and CTP, showing that these substrates could compete for the substrate binding site of the carrier (Figure 3A). Membrane vesicles were loaded with these substrates for an exchange reaction with radio-labeled ADP to investigate whether these substrates were not only binding, but also were actually transported by the carrier. The transport assays show that ATP and ADP and, to a lesser extent, AMP are the preferred substrates (Figure 3B). Classic mitochondrial ADP/ATP carriers also transport ADP and ATP, but not AMP [18]. High concentrations of phosphate are able to prevent the binding of nucleotide (Figure 3A), but this substrate is not translocated in exchange for ADP (Figure 3B).

A distinctive characteristic of mitochondrial ADP/ATP carriers is their sensitivity to the specific inhibitors carboxyatractyloside and bongkrekic acid [19]. The mitosomal carrier was not inhibited by these compounds (Figures 4A and 4B). The binding of carboxy-atractyloside to the mitochondrial ADP/ATP carrier has recently been explained in structural terms [9]. The residues that are important for binding of the inhibitor are not conserved in the *Entamoeba* carrier (Figures 1A and 1B).

Mitochondrial ADP/ATP carriers use an electrogenic transport mechanism for adenine nucleotide exchange [20]: $ATP^{4-}$ is exchanged for $ATP^{3-}$, resulting in a net transport of one negative charge across the membrane. Therefore, mitochondrial ADP/ATP exchange is driven by the concentration gradients of the substrates *and* the mitochondrial positive-outside membrane potential generated by the electron transport chain. An artificially generated negative-inside membrane potential reduced the uptake of ATP in exchange for ADP by the yeast ADP/ATP carrier 3 in *L. lactis* membrane vesicles—as expected for electrogenic transport (Figure 4C). *Entamoeba* lacks an electron transfer chain [21] and so is unable to generate a membrane potential by this means, raising the question of how its carrier functions. The membrane potential had no effect on ADP/ATP exchange by the *Entamoeba* carrier (Figure 4D), showing that exchange is electroneutral. The electroneutral exchange of ADP for ATP suggests that a posi-

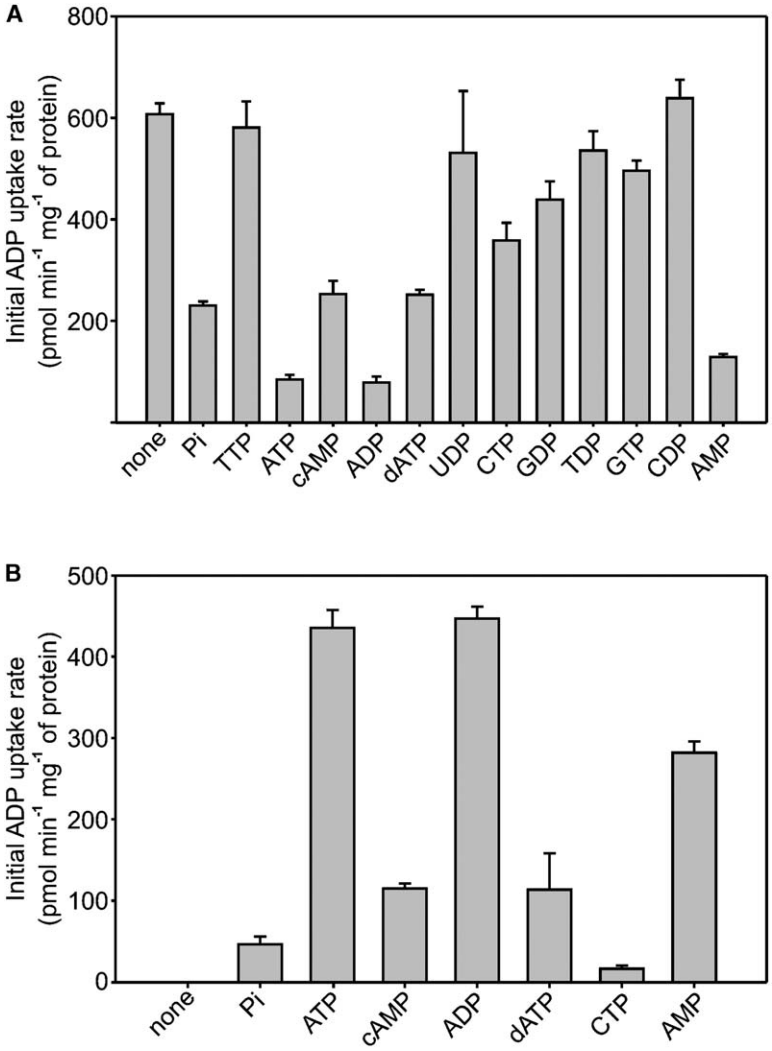


Figure 3. Substrate Specificity of the Mitosomal Carrier as Determined by Competition (A) and Active Transport (B)

(A) Initial uptake rate of radio-labeled ADP in the presence or absence of a 3,333-fold higher concentration of the indicated nonlabeled compounds.

(B) Initial uptake rate of radio-labeled ADP into fused membrane vesicles that were preloaded with different substrates. Only when the substrates are transported will exchange occur. High concentrations of phosphate are able to prevent the binding of nucleotide (A), but Pi is not translocated (B). In both types of experiments (A and B), membrane vesicles were loaded with 2 mM substrate and diluted 200-fold in a buffer containing 0.6 μM [$^{14}$C]-ADP. The initial uptake rates were calculated from the accumulation of radio-labeled ADP after 20 s as determined by scintillation counting after removal of external radio-labeled substrate by filtration.

tive counter ion is transported to compensate for the charge difference, but the type of ion has not been identified. The peroxisomal AMP/ATP carrier [22] and the mitochondrial GDP/GTP carrier [23] both use protons as counter ions for electron neutral exchange of nucleotides, and this may be the case for the mitosomal carrier too.

## Phylogenetic Analysis of the *Entamoeba* MCF Protein

We carried out a phylogenetic analysis of known MCF subfamilies (Supplemental Data). The *Entamoeba* protein formed a cluster together with uncharacterized homologs from the aerobic slime mold *Dictyostelium discoideum*, a close relative of *Entamoeba* [24]. Classic mitochondrial ADP/ATP carriers formed a separate cluster containing another homolog from *Dictyostelium*. The unfinished *Dictyostelium* genome contains 31 different mitochondrial carriers, representing most of the recognized MCF subfamilies [25, 26] from animals, plants, and protists. Recent phylogenetic analyses [27, 28] suggest that eukaryotes can be divided into two "supergroups" comprising animals, fungi, and amoebae such as *Entamoeba* and *Dictyostelium*, or plants, algae, and diverse protists including *Plasmodium* and *Trypanosoma*. Gene fusion data [28] suggest that the root of the eukaryotic tree is between these two supergroups. Because most MCF subfamilies occur on both sides of this rooted tree, the ancestral organelle must have already contained a broad repertoire of MCF members. *Entamoeba* has subsequently lost all but one of these carriers during its adaptation to a parasitic and anaerobic lifestyle.

## Concluding Remarks

The *Entamoeba* mitosome has reduced its transport capacity mediated by MCF members to an unprecedented degree and must therefore carry out only a limited subset of mitochondrial functions known from yeast mitochondria. Consistent with this hypothesis are biochemical data that suggest that *Entamoeba* lacks key mitochondrial pathways and that energy metabolism is cytosolic [21, 29]. Mitochondrial chaperonin 60—the only other protein currently known to localize

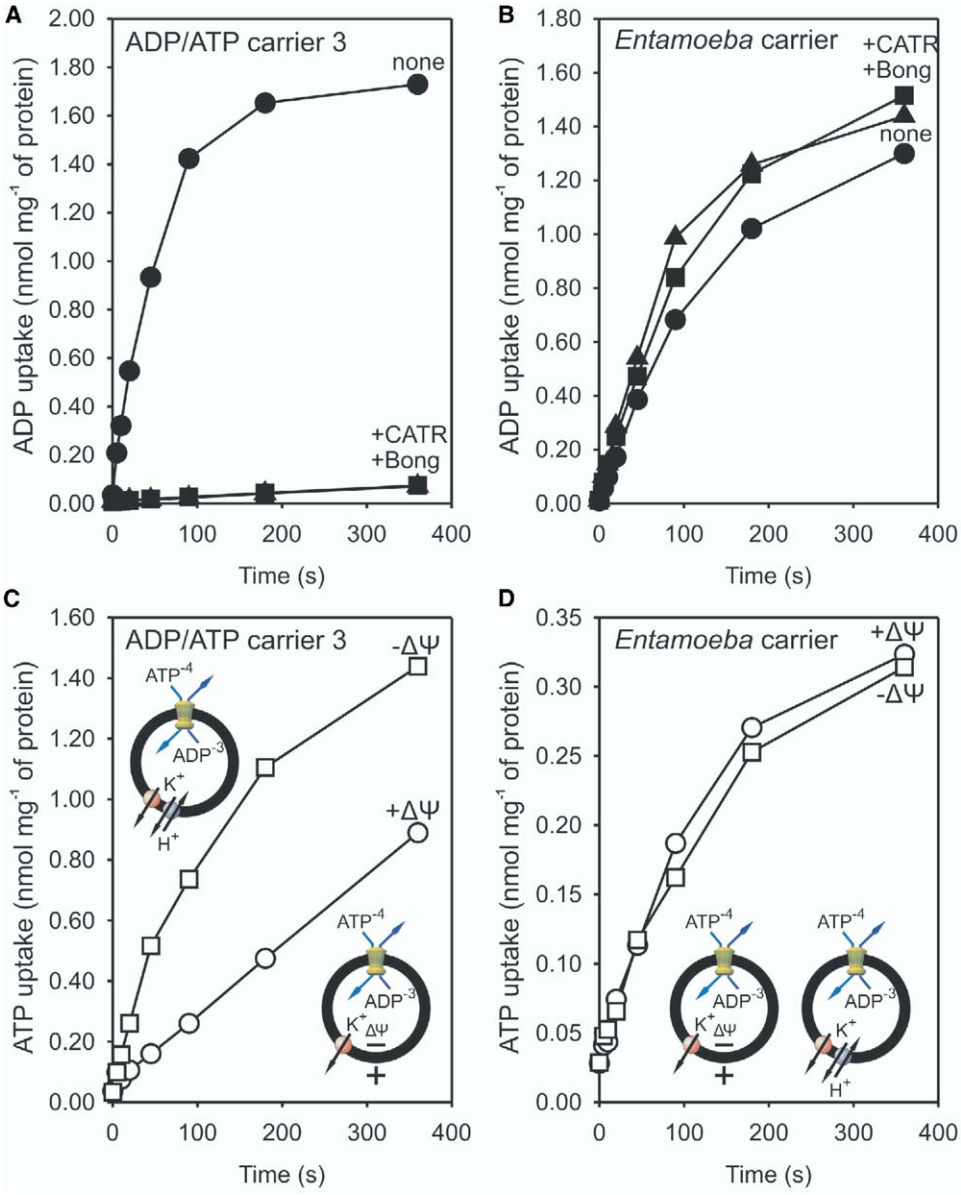


Figure 4. Inhibitor Specificity and Driving Forces of the Mitosomal Carrier and Yeast ADP/ATP Carrier 3

(A) Fused membranes of the strain expressing the yeast ADP/ATP carrier 3 and (B) the *Entamoeba* carrier were incubated for 30 min with the specific inhibitors carboxyatractyloside (closed triangles) and bongkrekic acid (closed squares) at concentrations of 20 μM and 5 μM, respectively. Membranes were loaded with 5 mM ADP, and the external ADP was removed by gel filtration. The exchange was initiated by diluting the membrane vesicles 3-fold in buffer containing 0.65 μM [$^{14}$C]-ADP.

(C and D) Artificial gradients were generated in the presence of valinomycin to determine the influence of the membrane potential on the adenine nucleotide exchange by the yeast ADP/ATP carrier and the *Entamoeba* carrier. Fused membrane vesicles of the two strains were prepared in buffer containing 50 mM Tris (pH 7.0), 200 mM KCl, and 5 mM ADP. The membranes were concentrated and diluted 200-fold in buffer containing 50 mM Tris (pH 7.0) with 200 mM NaCl, 0.7 μM [$^{14}$C]-ATP, and 14 μM valinomycin (open circles), which generates a transient negative-side membrane potential by valinomycin-facilitated efflux of $K^+$ down the concentration gradient. In a separate experiment, the generated membrane potential was dissipated by the addition of 1 μM nigericin to the dilution buffer (open squares), which dissipates the potential by nigericin-facilitated exchange of $K^+$ ions for protons. The accumulated radio-labeled ATP was determined by scintillation counting after removal of external radio-labeled substrate by filtration.

to the mitosome—requires ATP to fold nuclear-encoded proteins after import into mitochondria [30]. Our data suggest that the novel ADP/ATP carrier could supply the ATP for this, and any other, energy-requiring organelle process. Recent findings suggest that all eukaryotes still contain a mitochondrial homolog [1, 3–6], even when they lack oxidative phosphorylation and an organelle genome [31]. These discoveries suggest that mitochondrial homologs may be essential for the eukaryotic cell. The highly simplified *Entamoeba* mito-

some and the recently completed *Entamoeba* genome will provide valuable tools toward identifying why this might be so.

### Supplemental Data

Supplemental Data including detailed Experimental Procedures and four supplemental figures are available at http://www.current-biology.com/cgi/content/full/15/8/737/DC1/.

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