Mitochondrial evolution entailed the origin of protein import machinery that allows nuclear-encoded proteins to be targeted to the organelle, as well as the origin of cleavable N-terminal targeting sequences (NTS) that allow efficient sorting and import of matrix proteins. In hydrogenosomes and mitosomes, reduced forms of mitochondria with reduced proteomes, NTS-independent targeting of matrix proteins is known. Here, we studied the cellular localization of two glycolytic enzymes in the anaerobic pathogen *Trichomonas vaginalis*: PPi-dependent phosphofructokinase (TvPPi-PFK), which is the main glycolytic PFK activity of the protist, and ATP-dependent PFK (TvATP-PFK), the function of which is less clear. TvPPi-PFK was detected predominantly in the cytosol, as expected, while all four TvATP-PFK paralogues were imported into *T. vaginalis* hydrogenosomes, although none of them possesses an NTS. The heterologous expression of *TvATP-PFK* in *Saccharomyces cerevisiae* revealed an intrinsic capability of the protein to be recognized and imported into yeast mitochondria, whereas yeast ATP-PFK resides in the cytosol. TvATP-PFK consists of only a catalytic domain, similar to “short” bacterial enzymes, while ScATP-PFK includes an N-terminal extension, a catalytic domain, and a C-terminal regulatory domain. Expression of the catalytic domain of ScATP-PFK and short *Escherichia coli* ATP-PFK in *T. vaginalis* resulted in their partial delivery to hydrogenosomes. These results indicate that *TvATP-PFK* and the homologous ATP-PFKs possess internal structural targeting information that is recognized by the hydrogenosomal import machinery. From an evolutionary perspective, the predisposition of ancient ATP-PFK to be recognized and imported into hydrogenosomes might be a relic from the early phases of organelle evolution.

The transition of the mitochondrion into an ATP-producing organelle was the crucial event at the eukaryote origin (1). ATP synthesis in eukaryotes is typically compartmentalized, with glycolysis in the cytosol and pyruvate oxidation in the mitochondria, which is linked to highly efficient oxidative phosphorylation (1, 2). In protists, however, there are notable exceptions to the usual scheme regarding both glycolysis and pyruvate oxidation. In *Trichomonas vaginalis* and other eukaryotes that possess an anaerobic form of mitochondria called hydrogenosomes, pyruvate is oxidized within the organelle via less efficient anaerobic fermentation (3). *Giardia intestinalis*, *Entamoeba histolytica*, and other eukaryotes possess a reduced form of mitochondria called mitosomes that do not produce ATP at all (4). In these organisms, pyruvate oxidation takes place exclusively in the cytosol (1). In kinetoplastids, glycolysis is compartmentalized in specialized microbodies called glycosomes (5). In some green algae, the first half of the glycolytic pathway is localized in the chloroplast (6, 7), while in the diatom *Phaeodactylum tricornutum* and other stramenopiles, several glycolytic enzymes are targeted to multiple compartments, such as the cytosol, plastids, and mitochondria (8, 9).

A particularly vexing case of compartmentalization involves *T. vaginalis* phosphofructokinase (PFK). In *Trichomonas*, glycolysis proceeds via a pyrophosphate (PPi)-dependent phosphofructokinase (PP-PFK) (10), an enzyme that is generally rare in eukaryotes, albeit typical in plants (11). Therefore, it was surprising that genes for ATP-dependent phosphofructokinase (ATP-PFK) turned up in the *Trichomonas* genome (12). Furthermore, peptides of the expressed protein were found in the hydrogenosomal proteome (13–15), although the exact topology of hydrogenosome-associated *T. vaginalis* ATP-PFK (TvATP-PFK) remains unclear (13, 15). PPi-PFK and ATP-PFK share an evolutionary origin (16, 17). In bacteria, ATP-PFK is a homo-oligomeric enzyme that is formed by ~35-kDa subunits (18). In opisthokonts, ATP-PFK underwent gene duplication and fusion events, resulting in an ~90-kDa protein with an N-terminal catalytic domain and a C-terminal regulatory domain (19). The PPi-PFK protein forms homo- or, in plants, heterotetramers of ~40- to 60-kDa subunits, and in Apicomplexa, the two subunits are fused to a protein of ~140 kDa (20). The advantage of using PPi-PFK rather than ATP-PFK in glycolysis lies in the increased yield of ATP due to the replacement of ATP with PPi as a phosphate donor in the phosphorylation of fructose-6-phosphate (3). This is particularly important for *T. vaginalis* and other anaerobes with energy metabolism based mainly on glycolysis (10).

In most eukaryotes, the N-terminal targeting sequences (NTS) are required for the delivery of nuclear-encoded proteins into the mitochondrial matrix, whereas the NTS-independent pathway is mainly involved in the routing of proteins into the outer and inner mitochondrial membranes and the intermembrane space. NTS are typically 15 to 55 residues in length and form a positively

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charged amphipathic α-helix (21). Upon preprotein delivery into the matrix by the outer (TOM) and inner (TIM) membrane translocases, the NTS is removed by a heterodimeric zinc-dependent mitochondrial processing peptidase (MPP) (22). Proteins routed by the NTS-independent pathway possess either a single or multiple internal targeting signals (ITS) (23). In Saccharomyces cerevisiae and human mitochondria, the components and mechanisms of protein import via the NTS-dependent pathway are well characterized (23), whereas less is known about protein import in hydrogenosomes. The NTS-dependent mechanism is present in hydrogenosomes and mitoribosomes (4, 24, 25), but a few studies have also reported NTS-independent import into the hydrogenosomes of T. vaginalis (26, 27, 58).

Interestingly, there are four ~35-kDa TvATP-PFK proteins encoded in the T. vaginalis genome, none of which possesses an NTS. The multiple copies preclude the generation ofTvATP-PFK knockouts with current Trichomonas tools to study their functions, which remain mysterious. To clarify the localization and exact organellar topology of T. vaginalis hydrogenosomes, we assessed whether the homologous catalytic domain of yeast ATP-PFK could be recognized as a substrate for NTS-independent import into isolated hydrogenosomes, and tested whether TvATP-PFK could be recognized as a substrate for NTS-independent import into yeast mitochondria. Conversely, we assessed whether the homologous catalytic domain of yeast ATP-PFK, as well as ~35-kDa Escherichia coli ATP-PFK (EcATP-PFK), showed a tendency to be imported into hydrogenosomes when expressed in T. vaginalis.

**Materials and Methods**

T. vaginalis strain T1 (provided by J.-H. Tai, Institute of Biomedical Sciences, Taipei, Taiwan) was grown in Diamond’s tryptone-yeast extract-maltose (TYM) medium supplemented with 10% (vol/vol) heat-inactivated horse serum. **Phylogenetic analyses.** The sequences of ATP-PFK and PPi-PFK genes when expressed in transformed T. vaginalis cells using immunofluorescence microscopy and cell fractionation, characterized the ATP dependence ofTvATP-PFK import into isolated hydrogenosomes, and tested whether TvATP-PFK could be recognized as a substrate for NTS-independent import into yeast mitochondria. Conversely, we assessed whether the homologous catalytic domain of yeast ATP-PFK, as well as ~35-kDa Escherichia coli ATP-PFK (EcATP-PFK), showed a tendency to be imported into hydrogenosomes when expressed in T. vaginalis.

**Preparation of Cellular Fractions.** Highly purified hydrogenosomes were obtained from T. vaginalis total cell lysates by differential and Percoll gradient centrifugation as described previously (35). The cytosolic fraction was isolated according to the method of Sukat et al. (35) and subsequently centrifuged at 190,000 × g (the high-speed cytosolic fraction). Mitochondria of S. cerevisiae were isolated from the yeast according to the method of Gregg et al. (39).

**Preparation of Radiolabeled Precursor Proteins.** The TvATP-PFK1 gene was cloned into the modified psp64 poly(A) plasmid, which enables serted genes with C-terminal green fluorescent protein (GFP) in yeasts. Transformed trichomonads and S. cerevisiae cells were selected as previously described (33, 34). The primers that were used for amplification and cloning of the selected genes into the pTagVag2 and pYES2/CT plasmids are shown in the supplemental material.

The pTagVag2 plasmid allows expression of the inserted genes under the control of the T. vaginalis hydrogenosomal α-subunit succinylo-coenzyme A (CoA) synthetase (SCSα) gene promoter (33). Alternatively, we used native promoters of selected genes instead of the SCSα promoter. The selected genes were amplified by PCR with 300 bp of upstream noncoding sequences and inserted into the pTagVag2 plasmid with a deleted SCSα promoter (pTagVagN). The primers used to amplify and clone the selected genes with their native promoters are shown in the supplemental material. **Immunofluorescence microscopy.** Episomally expressed recombinant proteins were detected in trichomonads using a monoclonal mouse anti-HA antibody (35). In double-labeling experiments, hydrogenosomal malic enzyme was detected using a rabbit polyclonal antibody (36). A secondary Alexa Fluor 488 (green) donkey anti-mouse antibody and Al- exa Fluor 594 (red) donkey anti-rabbit antibody were used for visualization of target proteins. The cells were examined using an Olympus Cell-R IX81 microscope system. The acquired images were processed using ImageJ software (version 1.4d) (http://rsbweb.nih.gov/ij/). In S. cerevisiae cells, episomally expressed recombinant proteins with GFP were detected and examined as described above. In double-labeling experiment, mitochondria were detected with MitoTracker dye (Invitrogen).

**Enzyme assays.** ATP-PFK activity was determined in the glycolytic direction using a continuous spectrophotometric assay according to the method of Chi et al. (37) with some modifications. The assay mixture for ATP-PFK consisted of 2 ml of 100 mM HEPES, 50 mM KCl, 3 mM MgCl2, 1 mM EDTA, pH 7.0, buffer; 1 mM ATP; 20 mM fructose-6-phosphate; 0.15 to 0.20 mM NADH; 2 to 3 U each of aldolase, triosephosphate isomerase, and glycerol-3-phosphate dehydrogenase (Sigma-Aldrich); and 0.05% (vol/vol) Triton X-100 (ATP-PFK assay buffer). The assay was performed in 1-cm anaerobic cuvettes. The reaction was started by alternatively adding ATP, fructose-6-phosphate, auxiliary enzymes, or protein sample to the assay mixture, and the reaction was monitored as a decrease in the absorbance of NADH at 340 nm using a Shimadzu UV-2600 spectrophotometer. PPi-PFK activity was determined as previously described (38). The protein concentrations in the subcellular fractions of T. vaginalis were determined by the Lowry protein assay.

**Protease protection assay.** Aliquots of intact hydrogenosomes (3 mg) were resuspended in 1 ml of 1 X ST buffer (250 mM sucrose, 10 mM Tris, pH 7.8, 0.5 mM KCl) supplemented with protease inhibitor cocktail tablets (Roche Complete, EDTA free). Trypsin (Sigma) was added to a final concentration of 200 μg/ml, and the samples were incubated at 37°C for 30 min. After incubation, the trypsin activity was stopped by the addition of soybean inhibitors (5 mg/ml), and the samples were analyzed by immunoblotting with a monoclonal mouse anti-HA antibody. Aliquots of intact mitochondria (1 mg) were resuspended in 1 ml of SEM buffer (1 mM MOPS [morpholinepropanesulfonic acid]-KOH, pH 7.2, 250 mM sucrose, 1 mM EDTA). Proteinase K (Sigma) was added to a final concentration of 50 μg/ml, and the samples were incubated at 37°C for 30 min. After incubation, the proteinase K activity was stopped by the addition of 250 μl of trichloroacetic acid. The samples were analyzed by immunoblotting with a monoclonal anti-GFP antibody (Pierce).

**Preparation of radiolabeled precursor proteins.** The TvATP-PFK1 gene was cloned into the modified psp64 poly(A) plasmid, which enables
in vitro mRNA synthesis from the inserted genes (Promega). The primers designed for PCR and cloning into the psp64 plasmid are described in the supplemental material. In vitro transcription was performed using the mMachine kit (Ambion). [35S]methionine-radiolabeled precursor protein was synthesized in vitro using the Flexi Rabbit Reticulocyte Lysate System (Promega).

In vitro import. Each in vitro import assay was performed in a reaction mixture that included 100 μl of import buffer (10 mM HEPES, pH 7.4, 250 mM sucrose, 2 mM KPi, pH 7.4, 25 mM KCl, 10 mM MgCl2, 0.5 mM EDTA, pH 8.0, 1 mM dithiothreitol [DTT], 10 mM ATP), 50 μl of cytosolic extract, 5 μl of radiolabeled precursor protein, and 5 μg of isolated hydrogenosomes. Apyrase (20 U/ml) was used for the import assay, which was conducted in the absence of ATP. The organelles were preincubated for 10 min at 25°C in import buffer with cytosolic extract, after which radiolabeled precursor protein was added to the assay mixture, and the mixture was incubated for 1, 10, and 60 min at 25°C. At each time point, the in vitro import was stopped by the addition of 100 μg/ml of proteinase K and placed on ice for 20 min. After incubation, the activity of proteinase K was inhibited by adding 2 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma). The hydrogenosomes were then washed in import buffer and solubilized in SDS loading buffer. To test the activity of proteinase K, a 60-min incubation of the protein import reaction mixture, the hydrogenosomes were dissolved with 0.5% (vol/vol) Triton X-100, followed by the addition of 100 μg/ml of proteinase K. Proteins in the supernatant were precipitated with methanol-chloroform and solubilized in SDS loading buffer. All of the samples were subjected to SDS-PAGE in a 13.5% separating gel. The gels were vacuum dried and exposed to X-ray films.

RESULTS

Phylogenetic analysis reveals the presence of PPi-PFK and the short type of ATP-PFK in T. vaginalis and other parbasilids. The T. vaginalis genome possesses 11 genes encoding phosphofructokinases, four of which encode “short” (∼35-kDa)-type ATP-dependent PKFs (TvATP-PFK1 to -4 [TVAG_293770, TVAG_496160, TVAG_462920, and TVAG_391760]) and seven of which encode PPi-dependent PKFs (TvPPi-PFK1 to -7 [TVAG_430830, TVAG_077440, TVAG_281070, TVAG_364620, TVAG_079260, TVAG_263690, and TVAG_335880]). A phylogenetic analysis of ATP-PFKs and PPi-PFKs revealed that Trichomonas TVaTP-PFK1 to -4 fall into the single robust clade T2, together with PKFs from other parbasilids (Fig. 1). The closest eukaryotic relatives of this clade are tandem-fusion PKFs from opisthokonts and amoebobzoans (clade E), as well as enzymes from prokaryotes (clades B1 and B2). The Trichomonas homologues TvPPi-PFK1 to -7 also form a clade with parbasalian sequences (Fig. 1). This parbasalian clade (clade T1) branches with enzymes from jakobids, heteroloboseans, and prokaryotes. The presence of both versions of the enzyme in other parbasilids suggests that both PPi-PFK and ATP-PFK were present in the parbasalian ancestor. The branching of the T. vaginalis sequences in several unrelated positions in both clades T1 and T2 indicates that the genes have undergone gene duplications and possibly gene losses within parbasilids. The specificity of both types of PKFs for either ATP or PPi has been ascribed to the amino acid residues at positions 104 and 124, respectively. Therefore, the ability of TvATP-PFK2 to bind ATP is uncertain. The expected amino acid residues at positions 104 and 124, respectively, are present in TvPPi-PFK1 and -3 to -6, whereas TvPPi-PFK2 and -7 contain glutamic acid and alanine residue at position 104, respectively (Fig. 2; see Fig. S2 in the supplemental material). Interestingly, scanning of the alignment of a broad range of sequences that were used for the phylogenetic analysis (Fig. 1) revealed the presence of paralogous genes with canonical G104 and K124 amino acid residues and with different residues at these positions in other parbasilids of clade T2 and in members of the Embryophyta, clade P. For example, serine residues at position 124 are also present in the putative ATP-PFKs of Trichomonas foetus and Histomonas meleagris (see Fig. S2 in the supplemental material). Moreover, the H. meleagris protein contains asparagine at position 104. These sequences, together with TvATP-PFK2 and -4, form the upper branch of clade T2 (Fig. 1). The unusual paralogues of Embryophyta PPi-PFK-like sequences contain threonine/isoleucine and valine at positions 104 and 124, respectively (see Fig. S2 in the supplemental material), and they are grouped in the upper Embryophyta branch of clade P (Fig. 1). The functions of plant PPi-PFK-like proteins are unknown (41).

Cellular localization of TvATP-PFK paralogues. The analysis of TvATP-PFK1 to -4 revealed an absence of sequence motifs thought to target precursors to hydrogenosomes. The TvATP-PFK sequences are colinear with their bacterial orthologues, lacking a predictable NTS and the cleavage site for the processing peptidase (Fig. 2). We found no internal motifs for subcellular targeting, and PSORT II predicted TvATP-PFKs to localize to the cytosol. The subcellular localization of TvATP-PFK1 to -4 was investigated by the transient expression of C-terminally HA-tagged proteins in T. vaginalis. Immunofluorescence microscopy revealed that recombinant TvATP-PFK1, -2, and -4 colocalized with malic enzyme, the hydrogenosomal marker protein (Fig. 3; see Fig. S1 in the supplemental material), which suggested that these three proteins were transported into the hydrogenosomal matrix (we were unable to detect any expression of TvATP-PFK3 after several independent rounds of transfection). The topology of TvATP-PFK1 was further tested by protease protection assays. The treatment of isolated organelles with trypsin had no effect on the TvATP-PFK1 signal in the Western blot analysis, and the signal disappeared only in response to treatment with detergent (Fig. 3B). This finding indicates that TvATP-PFK is imported into T. vaginalis hydrogenosomes and is not associated with the organelle surface.

Although the bioinformatics analysis did not predict the presence of a cleavable NTS, we cannot exclude the possibility that a noncleavable “cryptic” NTS signal might direct TvATP-PFK1 to hydrogenosomes. Therefore, we expressed a truncated version of TvATP-PFK1 that lacked the first 16 amino acid residues (aa) (double the size of the known NTS in Fdx1). The truncated TvATP-PFK1 was delivered to the hydrogenosomes as its complete form (Fig. 3). This result confirmed that import of TvATP-PFK1 into hydrogenosomes is NTS independent. The expression of TvPPi-PFK revealed a cytosolic localization of the enzyme, as expected (Fig. 3).
FIG 1  Phylogeny of ATP- and PPi-dependent PFKs. Shown is a maximum-likelihood (ML) tree of PFK (191 taxa and 340 sites). The numbers at the nodes indicate bootstrap values (BV)/posterior probabilities (PP). Only BV and PP greater than 50% and 0.9, respectively, are shown. Branches with BV of 95% are marked by black circles, and branches with PP of 0.95 are marked by thick lines. Substrate specificity, molecular mass, and subunit composition for clades are indicated. The names of eukaryotes are in brown, and those of prokaryotes are in black.
Next we investigated PP$_i$- and ATP-dependent PFK activities in cellular fractions of _T. vaginalis_. Under anaerobic conditions, we detected specific PP$_i$-PFK activity of 0.4 to 0.9 μmol min$^{-1}$ mg protein$^{-1}$ in the high-speed cytosolic fraction. Percoll-purified hydrogenosomes contained a low specific activity (~0.008 to 0.020 μmol min$^{-1}$ mg protein$^{-1}$) of ATP-PFK. PP$_i$-PFK activity was not associated with the organelles. These results indicate that PP$_i$- and ATP-dependent PFK activities are present in _T. vaginalis_ in two distinct cellular compartments, in the cytosol and in hydrogenosomes, respectively. However, the hydrogenosomal (ATP-dependent) activity is dwarfed by the well-characterized cytosolic PP$_i$-dependent activity, raising questions about the role of the ATP-dependent activity, if any, in core energy metabolism.

**Expression of **TvATP-PFK1 and ferredoxin 1 under the control of native promoters. The _T. vaginalis_ SCso promoter is a strong endogenous promoter for transient expression (42). The unexpected localization of TvATP-PFK1 when transiently expressed under the control of the SCso promoter prompted us to test whether the promoter itself could influence the localization of the product. First, we tested SCso versus the native promoter (NP) by determining the cellular localization of Fdx1, a model pressed under the control of the SCS promoter. Therefore, we also assessed the localization of the recombinant TvATP-PFK1 expressed in _T. vaginalis_ under the control of its native TvATP-PFK1 promoter (Fig. 3). Immunofluorescence microscopy and Western blot analysis confirmed that under these conditions, TvATP-PFK1 was targeted into the hydrogenosomal matrix (Fig. 3).

**In vitro import of TvATP-PFK1 into hydrogenosomes.** TvATP-PFK1 import into hydrogenosomes was investigated using an *in vitro* import system. TvATP-PFK1 labeled with $^{35}$S was incubated with hydrogenosomes in import buffer supplemented with ATP and cytosolic extract for 0 to 60 min. After the incubation, the hydrogenosomes were treated with proteinase K to remove labeled proteins that were not imported into the organelles. These experiments revealed the time-dependent accumulation of radio-labeled TvATP-PFK1 within isolated hydrogenosomes (Fig. 5). Furthermore, we investigated whether ATP was necessary for import. When the import assay was supplemented with apyrase (20 U/ml), which converts ATP to AMP and pyrophosphate, no import of TvATP-PFK1 was observed (Fig. 5). This result indicates that NTS-independent import of TvATP-PFK1 requires ATP.

TvATP-PFK1 is recognized and imported into yeast mitochondria. It has been demonstrated that mitochondria and hydrogenosomes employ a common mode of NTS-dependent protein import (24). Thus, we were curious whether TvATP-PFK1 possesses an NTS-independent signal that is recognized by the protein import machinery of yeast mitochondria. We expressed TvATP-PFK1 with a C-terminal GFP tag in _S. cerevisiae_. Immunofluorescence microscopy showed that the GFP fusion protein colocalized with the mitochondrial marker MitoTracker (Fig. 6). A protease protection assay using isolated yeast mitochondria revealed that TvATP-PFK1 was imported into the organelle and excluded the possibility that the protein was associated with the mitochondrial surface. Cytochrome oxidase subunit VI was used as a control inner membrane protein. ScATP-PFK consists of an N-terminal extension of 200 aa, a catalytic domain of 359 aa, and a C-terminal regulatory domain (423 aa). When we expressed a truncated form that lacked the C-terminal regulatory domain (423 aa), the complete Fdx1 protein was imported into hydrogenosomes; however, Fdx1 with a deleted NTS remained in the cytosol (Fig. 4). It thus appears that the nature of the promoter that is used for protein expression may affect protein localization. In the case of Fdx1, the ITS is apparently not sufficient to deliver the protein into the organelles when the protein is expressed without NTS (ΔFdx1) under the control of the native promoter. Therefore, we also assessed the localization of the recombinant TvATP-PFK1 expressed in _T. vaginalis_ under the control of its native TvATP-PFK1 promoter (Fig. 3). Immunofluorescence microscopy and Western blot analysis confirmed that under these conditions, TvATP-PFK1 was targeted into the hydrogenosomal matrix (Fig. 3).
pressed the catalytic domain of ScATP-PFK, which is homologous to that of TvATP-PFK (ΔN1/2ScPFK) alone. Interestingly, although some ΔN1/2ScPFK signal was still observed in the cytosol, a significant portion was now also associated with the yeast mitochondrial membrane, as demonstrated by a protease protection assay (Fig. 6).

Collectively, these experiments show that TvATP-PFK1 possesses a targeting signal that is recognized by yeast mitochondria.
The complete ScATP-PFK is retained in the cytosol, but the catalytic portion of ScATP-PFK displays mitochondrial membrane affinity.

**Cellular localization of heterologous ATP-PFKs in *T. vaginalis***. We tested whether the hydrogenosomal protein import machinery can import heterologous ATP-PFKs. When we expressed complete ScATP-PFK in *T. vaginalis* under the control of the TvATP-PFK1 promoter, immunofluorescence microscopy revealed predominantly cytosolic localization of the protein, although the protein partially localized to hydrogenosomes (Fig. 7).
The expression of 1/2ScPFK revealed that the N-terminal half of ScATP-PFK was mainly associated with hydrogenosomes; however, the hydrogenosomal labeling was rather irregular in comparison to the labeling of malic enzyme, which was used as a control matrix protein. Western blot analysis of cellular fractions confirmed that both ScATP-PFK and 1/2ScPFK were present in the cytosolic fractions (low- and high-speed cytosolic fractions). Parts of both proteins were also associated with the hydrogenosomal fractions; however, the signals disappeared after trypsin treatment. When we expressed only the catalytic part of the yeast enzyme lacking the negatively charged N-terminal sequence (ΔN1/2ScPFK), a significant portion of the protein appeared inside the hydrogenosomes (Fig. 7). Next, we were interested in whether the targeting information is also present in short E. coli ATP-PFK orthologues that display 42% amino acid sequence identity with TvATP-PFKs. Thus, we expressed EcATP-PFK under the control of the TvATP-PFK1 promoter. Under these conditions, the E. coli protein was detected in the cytosol, and in part, it was associated with the hydrogenosomal surface (Fig. 7). However, when expressed under the SCSα promoter, a significant part of the protein was imported into the hydrogenosomes.

**DISCUSSION**

We investigated the cellular localization and NTS-independent import of TvATP-PFK into T. vaginalis hydrogenosomes. The parasite expresses both PPi- and ATP-dependent enzymes, which are compartmentalized in the cytosol and hydrogenosomes, respectively. The classical PPi-dependent activity of the parasite is about 50-fold higher than the newly characterized ATP-dependent activity, rendering the metabolic significance of the latter unclear. A phylogenetic analysis revealed that both types of PFKs are present across the parabasalids sampled so far. TvATP-PFK corresponds to a "short" ~35-kDa form of bacterial PFK that consists of only a catalytic domain, whereas the C-terminal regulatory domain typical of opisthokont ATP-PFKs is lacking. The targeting of TvATP-PFK1 to hydrogenosomes appears to be a

![FIG 5 In vitro import of TvATP-PFK1 into hydrogenosomes. In vitro-synthesized 35S-radiolabeled TvATP-PFK1 protein was incubated with isolated hydrogenosomes in import buffer at 25°C for 1, 10, and 60 min. At each time point, surface-associated proteins were degraded with proteinase K. Radiolabeled precursor was not imported in the absence of ATP (+ATP), depleted by addition of apyrase. A control for proteinase K activity was performed by the addition of Triton X-100 to the sample after 60 min of protein import (+Tx100). P, radiolabeled TvATP-PFK1 precursor protein. The samples were analyzed by SDS-PAGE and autoradiography.](ec.asm.org)

![FIG 6 Cellular localization of TvATP-PFK1 and yeast ATP-PFK in S. cerevisiae. (A) Domain structure of the expressed proteins. N, N-terminal extension; C, catalytic domain; R, regulatory domain; GFP, green fluorescent protein tag. (B) Immunofluorescence microscopy. TvATP-PFK1 was expressed in yeasts with C-terminal GFP (green). Mitochondria were detected using MitoTracker dye (Invitrogen) (red). TvATP-PFK1, complete short T. vaginalis PFK; ScPFK, complete long yeast PFK; 1/2ScPFK, N-terminal extension (205 aa) and catalytic domain (359 aa) of ScPFK; ΔN1/2ScPFK, catalytic domain with deleted N-terminal extension. (C) Immunoblotting of subcellular fractions and protein protection assay. GFP-tagged proteins were detected using an anti-GFP antibody. Cytochrome oxidase subunit IV (CoxIV) was used as a mitochondrial marker, which was detected using a rabbit anti-CoxIV antibody. Ly, total cell lysate; Ct, cytosol; M, mitochondria; Tr, hydrogenosomes treated with trypsin; Tx, hydrogenosomal fraction treated with trypsin and Triton X-100.](ec.asm.org)
highly specific and ATP-dependent process, even though the protein is not predicted to possess a cleavable NTS, which is typical of hydrogenosomal matrix proteins (44, 45).

The replacement of ATP with PPi as a phosphate donor in the phosphorylation of fructose-6-phosphate allows an increased glycolytic ATP yield (3), conceivably a significant feature for a fermenting organism. Examples of organisms that express both PPi-PFK and ATP-PFK are rare. The actinomycete Amycolatopsis methanolica possesses both genes, but their expression depends strictly on the carbon source (46). Entamoeba histolytica possesses two genes for PPi-PFK orthologues; however, one of the gene products has been shown to utilize ATP instead of PPi, and it has been suggested that the two enzymes might be expressed during different life stages (37). In plants, PPi-PFK and ATP-PFK are both cytosolic enzymes with reciprocal expression responding to environmental perturbations (47). Whereas the expression of PPi-PFK is upregulated by anoxia or orthophosphate deficiency, ATP-PFK is downregulated under such conditions. The spatial separation in T. vaginalis of PPi-PFK and ATP-PFK to the cytosol and hydrogenosomes, respectively, could be an alternative solution to avoid interference between the two enzymes.

Specific targeting of TvATP-PFK to the organelle was demonstrated in vivo by episomal expression of tagged TvATP-PFK1 under SCSα and its native promoters, as well as the in vitro import of radiolabeled protein into isolated hydrogenosomes. Through the HA-tagged SCSα-H9251, products of four paralogous TvATP-PFK genes were immunoprecipitated from isolated hydrogenosomes and identified by mass spectrometry. Earlier proteomic studies suggested association of the glycolytic pathway, including TvATP-PFK, with the hydrogenosome (13, 15), which raises the question of whether glycolytic enzymes form functional protein complexes on the hydrogenosomal outer membrane, as has been shown for mitochondria. For example, in Arabidopsis thaliana, 5 to 10% of each glycolytic enzyme is associated with the outer mitochondrial surface. Mammalian and fish heart mitochondria bind hexokinase and ATP-PFK (48), which has been discussed in the context of an increased glycolytic rate under hypoxic conditions (49). However, in T. vaginalis, expression of seven glycolytic
enzymes, including PPi-PFK, showed exclusively cytosolic localization of these proteins (15, 26). Moreover, available cell fractionation studies of glyceraldehyde-3-phosphate dehydrogenase (50) and PPi-PFK (this study) indicated that the corresponding activities are not associated with the organelle. These data do not support the formation of functional glycolytic complexes at the hydrogenosomal membrane and make the interpretation of previous proteomic analysis problematic, although systematic studies of glycolytic enzyme activities in cellular fractions of T. vaginalis are currently lacking. The localization of TvATP-PFK in the hydrogenosomal matrix, as shown in this study, is new for trichomonads.

Organelar forms of ATP-PFK have been found in glycosomes (51) and chloroplasts (52) thus far, where ATP-PFK operates within a known biochemical context. Kinetoplastids catalyze the “upper” six glycolytic steps in glycosomes, exporting 3-phosphoglycerate to the cytosol. Microalgal, such as Chlamydomonas reinhardtii, possess four glycolytic enzymes that convert glucose to glyceraldehyde-3-phosphate in chloroplasts, whereas the rest of glycolysis is localized in the cytosol (7). The most complicated glycolytic network has been found in diatoms, such as P. tricornutum, in which the complete set of glycolytic enzymes is present in the cytosol; nine glycolytic enzymes, including ATP-PFK, catalyze the conversion of glucose-1-phosphate to pyruvate in the chloroplast, and five glycolytic enzymes convert glyceraldehyde-3-phosphate to pyruvate in the mitochondrion (8). In these organisms, the specific targeting of various glycolytic enzymes into the organelles is mediated by NTS (mitochondria), peroxisomal targeting signals (glycosomes), and plastid targeting signal (chloroplasts). The organelar TvATP-PFK found in T. vaginalis is unique with respect to three features: (i) it is a single glycolytic enzyme that is compartmentalized without apparent distal and proximal partners in the pathway, (ii) it is the only PFK that was observed to be imported into mitochondrion-related organelles, and (iii) the import into hydrogenosomes is mediated by ITS. The overall low hydrogenosomal ATP-PFK activity (approximately 2% of the PPi-dependent activity), together with the lack of organelar glycolytic partners, raises questions regarding the metabolic role of TvATP-PFK and whether another function, unrelated to glycolysis, might be a possible alternative. Various moonlighting functions have been suggested for ATP-PFK in eukaryotes and bacteria, such as participation in the microautophagy of peroxisomes (53), RNA processing and degradation (54), and surface binding of plasminogen (55) and mannan (56). In our view, however, none of these functions currently appear likely for TvATP-PFK.

Heterologous expression of TvATP-PFK1 in S. cerevisiae revealed that the trichomonad enzyme is imported into yeast mitochondria, in addition to hydrogenosomes. This result indicates that TvATP-PFK1 possesses a targeting signal that is recognized by the hydrogenosomal, as well as the mitochondrial, import machinery. From an evolutionary perspective, these data suggest that the “short” ancient ATP-PFK might be predisposed to being recognized and imported into mitochondria, which might be a relic from the early phases of mitochondrial evolution. If so, the evolving eukaryotic cell had not only to develop a mechanism for targeting nuclear-encoded proteins to mitochondria, but also to prevent the organelar translocation of some proteins, such as ATP-PFK, that are components of cytosolic pathways. Interestingly, unlike short bacterial ATP-PFK, eukaryotes frequently possess structurally modified long ATP-PFK that consists of catalytic and regulatory domains. In addition, the ATP-PFK of yeast and other fungi is equipped with a negatively charged N-terminal extension that may interfere with organellar import. Indeed, when we expressed the catalytic domain of ScATP-PFK with the N-terminal extension (1/2ScPFK) in T. vaginalis, the protein was not delivered to the hydrogenosomal matrix, indicating that the extension prevents translocation. However, the hydrogenosomal import machinery was able to recognize and partially import truncated yeast ScATP-PFK, consisting of only the catalytic domain (ΔN1/2ScPFK), and the short proteobacterial EcATP-PFK, which are both homologous to TvATP-PFK. These results are consistent with the idea that ancient ATP-PFKs were predisposed to target the organelle. They also support previous analysis of proteins encoded by E. coli that predicted the presence of mitochondrial targeting information in about 5% of bacterial proteins (57).

The cell localization studies performed need to be interpreted with caution. Import of EcATP-PFK was observed when the gene was expressed under a strong SCSa promoter, while expression under the TvATP-PFK1 promoter resulted in partial association of TvATP-PFK1 with the outer hydrogenosomal membrane. Similarly, we observed promoter-dependent variation in the cell localization of Fdx, which possesses both NTS and ITS. Although we cannot exclude the possibility that hydrogenosomal localization of proteins expressed under strong promoters reflects protein mislocalization, it has been shown previously that six glycolytic enzymes expressed under the SCSa promoter remained exclusively in the cytosol, as expected, which argues against protein mislocalization (26). Therefore, it is more likely that, in addition to ITS, a suitable level of protein is required for protein translocation into the hydrogenosomes, while proteins without ITS are not targeted to the organelle regardless of the protein level. Importantly, expression of ΔN1/2ScPFK under TvATP-PFK1 was sufficient for its partial translocation into hydrogenosomes.

In conclusion, we identified ATP-PFK in T. vaginalis that is efficiently delivered into mitochondria and hydrogenosomes via NTS-independent mechanisms. Although NTS-independent targeting of membrane proteins is well documented, little is known about NTS-independent targeting of soluble proteins and the characters of multiple inner signals that are embedded within the protein structure (23, 58). The import of ATP-PFK into T. vaginalis hydrogenosomes can be used to investigate the molecular mechanisms that facilitate NTS-independent targeting and underpins the importance of internal targeting motifs that, in the case of PFK, are recognized in species spanning different eukaryotic supergroups. Intriguingly, the function of TvATP-PFK in T. vaginalis hydrogenosomes remains mysterious.

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