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Protein Import into Hydrogenosomes of *Trichomonas vaginalis* Involves both N-Terminal and Internal Targeting Signals: a Case Study of Thioredoxin Reductases▽†

Marek Mentel,‡ Verena Zimorski, Patrick Haferkamp,§ William Martin, and Katrin Henze*

Institute of Botany III, University of Duesseldorf, D-40225 Duesseldorf, Germany

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The parabasalian flagellate *Trichomonas vaginalis* harbors mitochondrion-related and H_2-producing organelles of anaerobic ATP synthesis, called hydrogenosomes, which harbor oxygen-sensitive enzymes essential to its pyruvate metabolism. In the human urogenital tract, however, *T. vaginalis* is regularly exposed to low oxygen concentrations and therefore must possess antioxidant systems protecting the organellar environment against the detrimental effects of molecular oxygen and reactive oxygen species. We have identified two closely related hydrogenosomal thioredoxin reductases (TrxRs), the hitherto-missing component of a thioredoxin-linked hydrogenosomal antioxidant system. One of the two hydrogenosomal TrxR isoforms, TrxRh1, carried an N-terminal extension resembling known hydrogenosomal targeting signals. Expression of hemagglutinin-tagged TrxRh1 in transfected *T. vaginalis* cells revealed that its N-terminal extension was necessary to import the protein into the organelles. The second hydrogenosomal TrxR isoform, TrxRh2, had no N-terminal targeting signal but was nonetheless efficiently targeted to hydrogenosomes. N-terminal presequences from hydrogenosomal proteins with known processing sites, i.e., the alpha subunit of succinyl coenzyme A synthetase (SCSα) and pyruvate:ferredoxin oxidoreductase A, were investigated for their ability to direct mature TrxRh1 to hydrogenosomes. Neither presequence directed TrxRh1 to hydrogenosomes, indicating that neither extension is, by itself, sufficient for hydrogenosomal targeting. Moreover, SCSα lacking its N-terminal extension was efficiently imported into hydrogenosomes, indicating that this extension is not required for import of this major hydrogenosomal protein. The finding that some hydrogenosomal enzymes require N-terminal signals for import but that in others the N-terminal extension is not necessary for targeting indicates the presence of additional targeting signals within the mature subunits of several hydrogenosome-localized proteins.

The parabasalian flagellate *Trichomonas vaginalis* is the causative agent of the most prevalent nonviral sexually transmitted disease in humans (34) and possesses hydrogenosomes, which are anaerobic forms of mitochondria (30, 34). Hydrogenosomes, like the mitochondria of aerobic eukaryotes, are involved in energy metabolism, but ATP is synthesized through substrate-level phosphorylation instead of oxidative phosphorylation (35). Although this fermentative energy metabolism in *T. vaginalis* hydrogenosomes relies on the highly oxygen-sensitive enzymes pyruvate:ferredoxin oxidoreductase (PFO) (53) and [Fe]-hydrogenase (39), *T. vaginalis* is frequently exposed to oxygen concentrations of up to 60 μM in its natural habitat on the vaginal surface (52). Furthermore, exposure to low oxygen concentrations on the order of 0.25 μM yields its maximum growth rates (40), owing to the ability of the organism to more rapidly establish a redox balance with NAD(P)H-oxidases. These enzymes have high activities and rapidly reduce O_2 to water, thereby affording the organism anaerobic growth conditions (31, 50), whereby at O_2 concentrations in the range of 50 to 250 μM, 5 to 10% of the O_2 consumed can be converted into H_2O_2 (9). Although NAD(P)H-oxidases (diaphorases) afford O_2-sensitive enzymes in *Trichomonas* protection against O_2 (48), they do not provide protection against reactive oxygen species such as the superoxide radical and hydrogen peroxide (H_2O_2). Detoxification of superoxide radicals in trichomonads is performed by iron-containing superoxide dismutase which is active in both the hydrogenosomal and cytosolic compartments (31, 25, 42). On the other hand, the analysis of thiols in whole cells of *T. vaginalis* indicated the absence of glutathione, and activities of peroxide-reducing enzymes, such as glutathione- and heme-dependent peroxidases, were not detectable (15). Catalase was shown to be present only in *Tritrichomonas foetus* and not in *T. vaginalis* (39). Such findings led to the suggestion that the lack of peroxide-reducing enzymes was the reason for the sensitivity of *T. vaginalis* to oxygen concentrations above physiological levels (15).

More recently, a thioredoxin-linked antioxidant system consisting of thiol peroxidase, thioredoxin, and thioredoxin reductase (TrxR) was identified, which is localized in the cytosol (10). The importance of this system as one of the most significant antioxidant defense mechanisms in *T. vaginalis* was shown by the upregulation of thioredoxin- and thioredoxin peroxidase-encoding genes in response to oxidative stress inducing environmental changes. Thioredoxin peroxidase belongs to a recently discovered thiol peroxidase family of proteins named

* Corresponding author. Mailing address: Institute of Botany III, Heinrich-Heine University of Duesseldorf, Universitaetsstrasse 1, 40225 Duesseldorf, Germany. Phone: 49-211-81-11378. Fax: 49-211-81-13554. E-mail: katrin.henze@uni-duesseldorf.de.

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‡ Present address: Department of Biochemistry, Faculty of Natural Sciences, Comenius University, Mlynská dolina CH-1, 842 15 Bratislava, Slovakia.

§ Present address: Molekulare Enzymtechnologie, Universität Duisburg-Essen, Campus Duisburg, Geibelstr. 41, 47057 Duisburg, Germany.

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peroxiredoxins, which use conserved cysteines at their active sites for catalysis (45). Peroxiredoxins are able to reduce hydrogen peroxide and alkyl peroxides as well as peroxynitrite with electrons provided by physiological thiols such as thioredoxin (19, 45). The third redox partner in the system, which serves as a reductant to oxidized thioredoxin, is low-molecular-weight TrxR. Components of a functionally related antioxidant protection system were recently found in the hydrogenosomes of *T. vaginalis*, where hydrogenosomal proteome screening identified two novel proteins with strong sequence similarity to two prokaryotic peroxidases, rubrerythrin and peroxiredoxin thiol peroxidase (41). A thioredoxin with an N-terminal extension showing strong similarity to hydrogenosomal targeting presequences was also found in the same study. Thus, two out of three components of the cytosolic thioredoxin-linked antioxidant system (10) were shown to also be present in the hydrogenosome (41), but the hydrogenosomal electron donor for thioredoxin was missing.

Here we report the identification of two hydrogenosomal TrxR isoforms, the hitherto-missing component of the hydrogenosomal thioredoxin-linked peroxidase antioxidant system, from *T. vaginalis*. The intracellular distribution of different TrxR isoforms observed in this study, together with the divergent N termini of these isoforms, led us to investigate the identity and importance of the targeting signals that direct hydrogenosomal TrxRs into the organelle in more detail. The hydrogenosomes of *T. vaginalis* lack a genome, and thus all hydrogenosomal proteins are encoded in the nuclear genome and must be imported into the organelle. This import seems to rely on translocation complexes similar to, but probably less complex than, those in mitochondria. No homologs to components of the protein import complex of the mitochondrial outer membrane (TOM) have so far been identified (8), but proteins homologous to Tim23 and Tim17, core components of the mitochondrial inner membrane translocase complex (TIM), as well as the protein import motor Pam18 of the mitochondrial matrix, are present in hydrogenosomes (12). Many hydrogenosome-targeted proteins carry short, cleavable, N-terminal extensions reminiscent of mitochondrial targeting presequences (5, 21, 27). These presequences carry recognizable cleavage sites which are processed by a hydrogenosomal processing peptidase, a homolog to mitochondrial processing peptidases (6).

Investigation of N-terminal presequences previously thought to be required to import proteins into the hydrogenosome in transfected *T. vaginalis* cells expressing hemagglutinin-tagged constructs in this study uncovered an unexpected plasticity among hydrogenosomal targeting signals in this pathogen.

MATERIALS AND METHODS

Strains and cultivation. *T. vaginalis* strain T1 (J.-H. Tai, Institute of Biomedical Sciences, Taipei, Taiwan) was grown in TYM medium at 37°C as described previously (33). *Escherichia coli* XL1-Blue MRF′ {Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^r)]} was used for cloning of genes of interest, and it was grown in LB medium containing ampicillin (100 μg/ml; Roth) at 37°C when transformed with the ampicillin resistance-bearing plasmid pTagVag2.

Cloning, N-terminal deletion, and transient expression of TrxR isoforms. Open reading frames of all five *TRXR* genes without the stop codon were amplified by PCR using genomic DNA of *T. vaginalis* as the template and sequence-specific primers for each gene (Table 1). N-terminally deleted genes were amplified from cloned full-length genes. Forward and reverse primers contained NdeI and BamHI restriction sites, respectively, for cloning the genes into the pTagVag2 expression vector (20). The only exception was TrxR_88289; because of an NdeI cutting site starting at position 710, only the first 708 nucleotides of the full-length 1,002-nucleotide-long open reading frame were amplified. After the BamHI restriction site, a dihemagglutinin tag (HA tag) was added in frame, followed by a stop codon. *T. vaginalis* T1 cells were electroporated (11, 28) with cloned constructs, and transformants were selected in TYM medium containing G418 (100 μg/ml; Roth).

TABLE 1. Oligonucleotides used in this study

Primer designation	Nucleotide sequence
TrxRh2 for	CTGACGCATATGTCGGGTGACATTGATTGGACTAAAGC
TrxRh2 rev	GACGTGGATCCGTCCGTTAAATATTTCTCAGC
TrxR L92 for	CTGACGCATATGCTTCCAATTTTACTTCCTTTCTTGC
TrxR L92 rev	GACGTGGATCCTTCTTGATTTTCTTCTGCGTCG
TrxR k96 for	CTGACGCATATGTCTGCTCAAGCATTCGATCTC
TrxR k96 rev	GACGTGGATCCGTCACTGAGATATCTCTCAGC
TrxR L88 for	CTGACGCATATGATGATATTTTGTTTTTTGTTTTTTGG
TrxR L88 rev-new	GACGTGGATCCTCCTGTAATGTTTTTGACAAC
TrxRh1 for	CTGACGCATATGTTTTCTATTATCTTTTTCTCTAG
TrxRh1 rev	GACGTGGATCCAATAGCTTTCACTTGTGATATATAC
TrxRh1 Δ10 fwd	CTGACGCATATGTACTCTACCGACGATGACG
TrxRh1 Δ36 fwd	CTGACGCATATGTGGAGTAAAGCGCCATTATAT
TrxRh1prePFO A	CTGACGCATATGCTCCGCAGCTTCTACTCTACCGACGATGACGATGATGAAATG
TrxRh1preSCSα	CTGACGCATATGCTCTCCTCTTCCTTCGAGCGCAACTACTCTACCGACGATGAC GATGATGAAATG
TrxRh2 Δ21 for	CTGACGCATATGGGTGGCAGCACAGCAGCTC
SCSα for	CTGACGCATATGCTCTCCTCTTCCTTCGAGCGC
SCSα rev	GACGTGGATCCGATCTTGCCCATTCTCTTCATC
SCSα Δ8 for	CTGACGCATATGCTCCACCAACCACTCCTTTTCATCG

Cell fractionation and protein concentration determination. Isolation of hydrogenosomes was based on the method described by Bradley et al. (5) with modifications (41). Whole-cell lysate (supernatant) was collected after grinding of the cells and the removal of unlysed cells, glass bead remains, membranes, and nuclei by centrifugation at 500 × *g* for 10 min at 4°C. The cytosolic fraction (supernatant) was obtained by subsequent centrifugation of the whole-cell lysate at 8,000 × *g* for 15 min at 4°C. Protein concentrations were determined with the Bradford assay kit (Bio-Rad) according to the manufacturer's instructions.

Enzyme assays. The activity of malic enzyme was assayed by monitoring the reduction of $NADP^+$ as described previously (26). Malate dehydrogenase activity was assayed in the direction of malate formation by observing the oxidation of NADH (46).

Western blot analysis. Samples (20 μg of proteins) for Western blot analysis were run on 12% resolving gels (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and blotted onto nitrocellulose membranes (Hybond-C Extra; Amersham Biosciences). Blots were washed (three times for 10 min each) in TBS (20 mM Tris-HCl [pH 7.5], 150 mM NaCl) and blocked for 1 hour in TBS containing 3% (wt/vol) bovine serum albumin. The blots were then incubated for 1 hour at room temperature, with subsequent overnight incubation at 4°C with mouse anti-HA antibodies (dilution 1:5,000; Sigma). Blots were washed as before and incubated with anti-mouse immunoglobulin G (heavy plus light chains)–horseradish peroxidase conjugate (ImmunoPure goat; dilution, 1:10,000; Pierce)

FIG. 1. Multiple alignment of the predicted amino acid sequences of *T. vaginalis* TrxRs. The deduced amino acid sequences of TrxRs were aligned using ClustalW (1.83). TrxR numbers reflect their identification numbers in the genome database (http://www.tigr.org). The conserved CATC motif of the active site redox centers of low-molecular-weight TrxRs is shaded in gray. Putative N-terminal targeting sequences are underlined.

in TBS containing 10% (wt/vol) dry milk powder for 2 to 3 h at room temperature. After subsequent washes in TBS, signals were visualized using SuperSignal West Pico peroxide substrate (Pierce) and Lumi-Film chemiluminescent detection film (Roche).

Neighbor-Net analysis. TrxR homologs were identified by BLAST searches at GenBank (http://www.ncbi.nlm.nih.gov/blast/). Protein sequences were aligned using ClustalW (1.83) (49). All gapped positions were removed from the alignment. The sequence similarity network was constructed with Neighbor-Net (7) and visualized with SplitsTree4 (23). Accession numbers of all protein sequences used in Neighbor-Net analysis are shown in Fig. S1 in the supplemental material.

RESULTS

Identification of TrxR genes in the *Trichomonas vaginalis* genome. Genes coding for TrxR were revealed by a BLAST search of the *T. vaginalis* genome database (http://www.tigr.org/) using the nucleotide sequence of cytosolic TrxR (10) as the query. Five homologs were identified, which are predicted to encode proteins ranging from 304 amino acids (10) to 352 amino acids and were thus classified as low-molecular-weight TrxRs (1, 37). When they were aligned by using ClustalW (1.83) (49), some differences among the proteins became evident (Fig. 1). Two of the putative TrxRs, including the one identified by Coombs et al. (10), were short isoforms with 304 (TrxR_96723, 96723.m00098) and 311 (TrxR_84394, 84394.m00089) amino acids. Three longer isoforms with 334 (TrxR_88289, 88289.m00178), 346 (TrxR_89667, 89667.m00077), and 352 (TrxR_92349, 92349.m00258) amino acids contained N-terminal extensions of different lengths (Fig. 1). Two of the short TrxRs and one N-terminally extended isoform carried the conserved CATC amino acid motif of the active-site redox center of low-molecular-weight TrxR (37), marking these proteins as putatively active TrxRs. The other two proteins diverged by only one amino acid from the consensus. All five TrxR-encoding genes of *T. vaginalis* are expressed as shown by the presence of the corresponding expressed sequence tags in the databases.

Subcellular localization of TrxR isoforms. The short TrxR isoform TrxR_96723 was shown by Coombs et al. (10) to be active in the soluble cytosolic fraction of *T. vaginalis* cells and for convenience is here designated TrxRc. Subcellular localization of the other four isoforms was performed to determine the identity of the hydrogenosomal TrxR isoenzyme(s) that remained to be identified, with TrxR_89667 and TrxR_92349 being the most likely candidates, as their N termini remotely resembled known hydrogenosomal targeting sequences. They carried a putative processing site at a suitable distance from

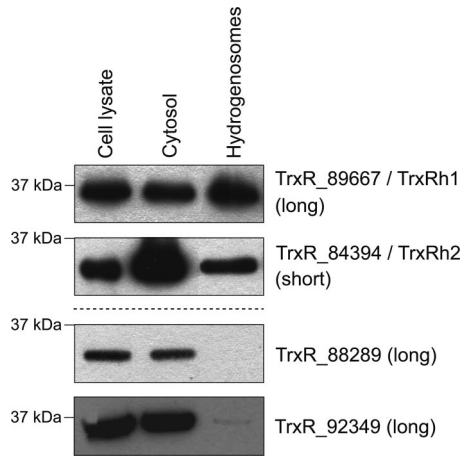

FIG. 2. Cellular localization of *T. vaginalis* TrxRs. Three cellular/subcellular fractions of *T. vaginalis* expressing HA-tagged TrxR isoforms were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequent Western blotting. The tagged TrxRs were detected with mouse anti-HA antibodies.

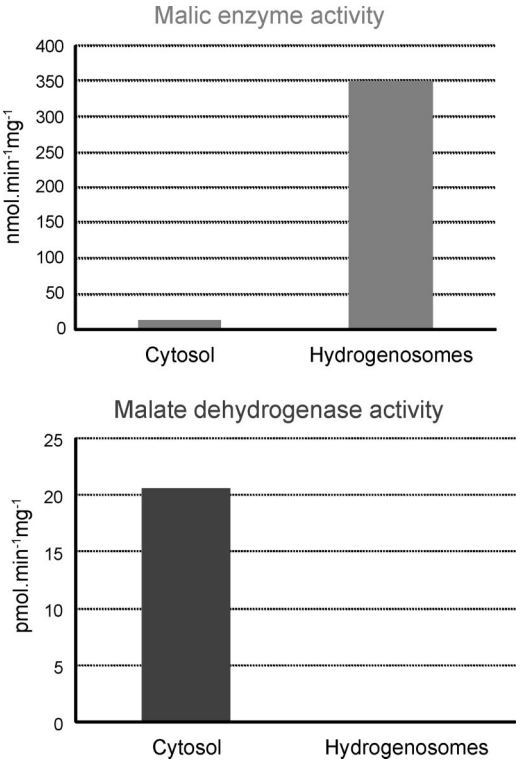

FIG. 3. Activities of marker enzymes in subcellular fractions of *T. vaginalis*. Activities of malate dehydrogenase (cytosolic marker) and malic enzyme (hydrogenosomal marker) in the cytosol and hydrogenosomes of *T. vaginalis* expressing HA-tagged TrxR_89667 (TrxRh1) are shown.

the N terminus and either started with the typical Met-Leu motif or were rich in Ser at the N terminus (Fig. 1) (5). *T. vaginalis* T1 cells were transformed with HA-tagged constructs of the four remaining TrxR genes in the vector pTagVag2. Three cellular/subcellular fractions, i.e., whole-cell lysate, cytosol (without hydrogenosomes), and hydrogenosomes, of the recombinant strains were subjected to Western blot analysis with antibodies against HA and analyzed for the presence of the HA tag (Fig. 2). As expected, the majority of TrxR_89667, henceforth designated TrxRh1, was imported into the hydrogenosomes, but a substantial amount of the protein remained in the cytosol. This result was reproducible in four individual experiments, indicating inefficient hydrogenosomal import and a putative dual localization of this protein. The marker enzyme controls, malic enzyme (hydrogenosomal marker) and malate dehydrogenase (cytosolic marker) (Fig. 3), showed that cross contamination between cytosolic and hydrogenosomal fractions was minute, such that leakage from hydrogenosomes during cell fractionation is insufficient to account for the cytosolic signal observed for TrxRh1.

The coding region of TrxR_84394 possesses, in comparison to TrxRc, an N-terminal extension of only seven amino acids with no resemblance to typical hydrogenosomal targeting sequences (Fig. 1). TrxR_84394 was also found in both subcellular fractions, but the bulk of TrxR_84394 HA-tagged protein remained in the cytosol and only a minor quantity was reproducibly directed to the hydrogenosomes (Fig. 2). From these results we concluded that either the short N-terminal extension of TrxR_84394, henceforth designated TrxRh2, had a weak targeting capacity or the protein had an internal signal that was able to direct the protein into the hydrogenosomes, albeit with low efficiency.

TrxR_92349 and TrxR_88289, the other two isoforms with N-terminal extensions (Fig. 1), were not imported into the hydrogenosomes (Fig. 2), indicating that these proteins possess neither N-terminal nor internal hydrogenosomal targeting signals.

TrxRh1 without an N-terminal extension stays in the cytosol. TrxRh1 was the only one of three TrxRs with substantial N-terminal extensions (Fig. 1) that was, at least partially, imported into the hydrogenosomes (Fig. 2). To test whether this presequence was involved in targeting TrxRh1 to hydrogenosomes of *T. vaginalis*, we examined the N terminus of TrxRh1 in more detail in transfected cells. As the first 10 amino acids had only vague similarity to previously recognized hydrogenosomal targeting signals (8, 18), we first removed the N-terminal 36-amino-acid extension relative to the cytosolic isoform TrxRc. This construct, TrxRh1-Δ36, remained exclusively in cytosol (Fig. 4a), indicating that this extension contains information that is necessary for organellar targeting. Hydrogenosomal targeting sequences are thought to be short, i.e., on the order of 10 amino acids, with Leu or Ser at the second position (8, 18) and with an Arg residue typically located at position −2 or −3 relative to the putative processing site of the N-terminal extensions (18). The first 11 amino acids of TrxRh1 lack Leu or Ser at position 2 but possess an Arg residue at position 9 (Fig. 1). This sequence was deleted in construct TrxRh1-Δ10, which also was not imported (Fig. 4a). This indicates that, though lacking some typical features, the N-terminal extension MFSI IFFSRFS is required for hydrogenosomal targeting of TrxRh1 and that its absence is not compensated for by an internal signal. It cannot be excluded, though, that an internal signal is necessary but not sufficient for import in the absence of that 11-amino-acid motif. As TrxRh2 was partially imported into

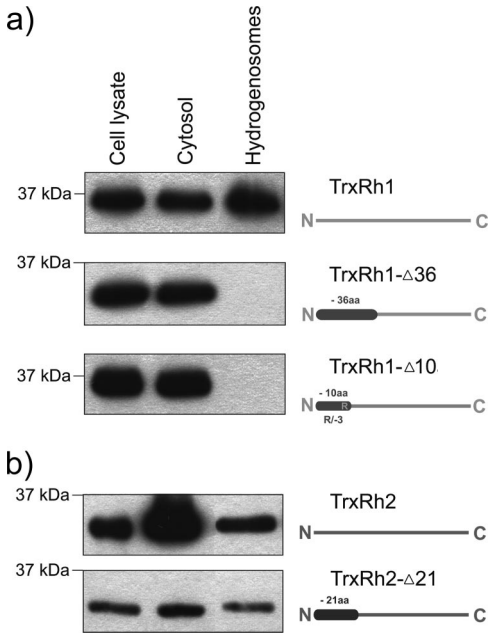

FIG. 4. Identification of hydrogenosomal targeting presequences of TrxRh1 and TrxRh2. (a) Subcellular localization of HA-tagged full-length TrxRh1 compared to HA-tagged N-terminally deleted TrxRh1-Δ36 and TrxRh1-Δ10. The arginine (R) position in the hydrogenosomal targeting signal of TrxRh1 is indicated. (b) Subcellular localization of HA-tagged full-length TrxRh2 compared to HA-tagged N-terminally deleted TrxRh2-Δ21. Deletions are not drawn in proportion.

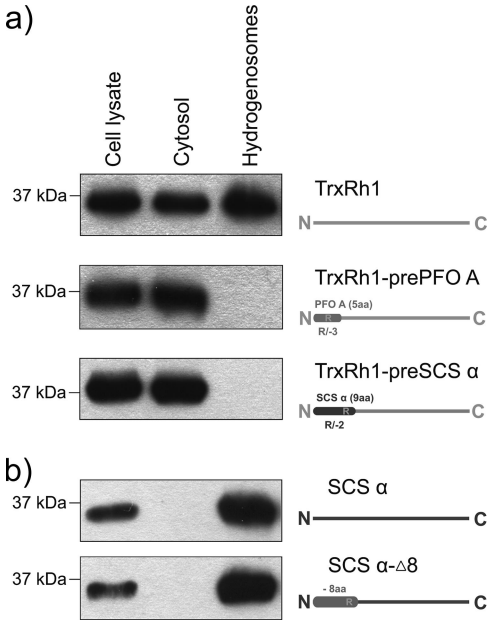

FIG. 5. Localization of TrxRh1 with N-terminal presequences of hydrogensomal proteins PFO A and SCSα. (a) Subcellular localization of HA-tagged full-length TrxRh1 compared to two HA-tagged constructs of TrxRh1 with the presequences of TrxRh1-prePFO A and TrxRh1-preSCSα. (b) Subcellular localization of HA-tagged full-length SCSα and SCSα-Δ8. R, arginine at position −2 relative to the processing site.

the hydrogenosomes, though having only a very short N-terminal extension without any resemblance to known hydrogenosomal targeting sequences, we examined the influence of its N terminus upon import. Since there was no obvious putative processing site in the first 34 amino acids of the N terminus of TrxR_84394, we removed the first 21 amino acids, which corresponded to approximately twice the characteristic presequence length of hydrogenosomal proteins in *T. vaginalis* and contained the first strongly conserved region of all five TrxR isoforms (Fig. 1). We were surprised to find that the distribution of TrxRh2-Δ21 between cytosol and hydrogenosomes did not differ markedly from that of the wild-type protein in that a minor amount of the protein still localized to the hydrogenosomes (Fig. 4b). Thus, in contrast to TrxRh1, which is not imported without the first 10 amino acids after methionine, TrxRh2 must contain an internal targeting signal that acts independently of an N-terminal targeting sequence. That the HA tag itself does not confer hydrogenosomal targeting is shown by TrxR_88289 and TrxR_92349, which remain cytosolic despite the presence of the HA tag (Fig. 2).

Localization of TrxRh1 with typical, processed hydrogenosomal targeting presequences. After identification of a hydrogenosomal targeting sequence (albeit an inefficient one) in TrxRh1, we investigated the localization of this protein under the influence of a presequence from a protein known from biochemical studies to be localized exclusively in the hydrogenosomes. For this purpose, the presequence MLRSF of PFO A (locus tag TVAG_198110) was chosen, because complete hydrogenosomal localization of the protein and the processing site of the N-terminal extension are known from earlier work (21). If this targeting signal is necessary and sufficient for import, it should guide TrxRh1 completely to the organelles. Surprisingly, TrxRh1 with its own presequence MFSIIFFSRFS exchanged for the presequence MLRSF of PFO A (TrxRh1-prePFO A) stayed entirely in the cytosol (Fig. 5a), indicating that it is not sufficient for import. As the presequence of PFO A is extremely short, i.e., only half of the length of the TrxRh1 presequence, the experiment was repeated using a different, previously characterized presequence with approximately the same length as that in TrxRh1. For this, we used the presequence MLSSSFERN of the alpha subunit of succinyl coenzyme A synthetase (SCSα) (locus tag TVAG_165340) because, as for PFO A, SCSα is localized to hydrogenosomes and the processing site is known (28). Again, this presumably "typical" presequence was also not sufficient to direct construct TrxRh1-preSCSα to hydrogenosomes (Fig. 5a). This indicates that the PFO A and SCSα presequences, though demonstrably processed upon import of their cognate precursor proteins, do not contain by themselves all information that is required to direct proteins to hydrogenosomes in *T. vaginalis*, and hence additional information for localization must be present in the sequences of the imported subunits.

The processed N-terminal presequence of SCSα is not necessary for hydrogenosomal targeting. The observations described above called into question the specificity and role of N-terminal sequences in *T. vaginalis* hydrogenosomal targeting and pointed to the presence of signals contained within the sequence of the mature subunit. To explore this possibility further, we removed the N-terminal presequence of SCSα at its

known processing site (SCSα-Δ8) and found that the mature HA-tagged subunit is still exclusively localized in the hydrogenosomes (Fig. 5b). We conclude that the presequence of SCSα, although processed (28), is not necessary to target the protein to the hydrogenosomes and that the mature SCSα subunit must have an internal targeting signal. Furthermore, the N-terminal sequence of SCSα is also not sufficient for targeting, because it does not substitute for the N terminus of TrxRh1, which is required for import (Fig. 5a). Hence the presequence of SCSα, though cleaved upon import, does not by itself contain any signals that specify import into the *T. vaginalis* hydrogenosome.

DISCUSSION

Peroxiredoxins, a recently discovered family of thiol peroxidases (24), are low-efficiency peroxidases that use thiols as electron donors. They are responsible for antioxidant defense in variety of organisms, including bacteria, yeasts, and trypanosomatids (19). Considering the lack of catalase (39) and glutathione (15) together with the absence of activities of peroxide-reducing enzymes (e.g., glutathione-dependent peroxidase) in *T. vaginalis*, it was expected that this human parasite should possess alternative means to deal with hydrogen peroxides and alkyl peroxides. Coombs et al. (10) identified a divergent thioredoxin-linked peroxiredoxin antioxidant system as the likely candidate for the principal antioxidant defense mechanism in the cytosol of *T. vaginalis*. Shortly thereafter, an analogous thiol-dependent antioxidant defense system was detected in hydrogenosomes of *T. vaginalis* (41). Along with peroxiredoxin thiol peroxidase, its reductant thioredoxin with an N-terminal extension strongly resembling presumed hydrogenosomal targeting presequences was found in the hydrogenosomal proteome (41). This predicted the presence of an electron donor to thioredoxin in hydrogenosomes.

The conventional electron donor for thioredoxin in thioredoxin-linked redox systems is TrxR (37). TrxRs found in eukaryotes fall into two distinct groups: (i) high-molecular-mass TrxR isoforms (~55 kDa) and (ii) low-molecular-mass TrxR isoforms (~35 kDa) (1). These two distinct forms have different structures and catalytic mechanisms and show a mutually exclusive distribution: high-molecular-mass TrxRs are found in animals and apicomplexan protists, while most of the other eukaryotic taxa possess the presumably ancestral low-molecular-mass TrxR isoform (17). The *T. vaginalis* TrxR identified by Coombs et al. (10) clearly belongs to the latter class. We found five open reading frames corresponding to homologs of low-molecular-mass TrxRs in the *T. vaginalis* genome. Though they were very similar to one another, some differences were evident. Only three *T. vaginalis* TrxR isoforms, including that identified by Coombs et al. (10), possessed the fully conserved active-site amino acid sequence CATC (Fig. 1), and three had N-terminal extensions that could possibly serve as hydrogenosomal targeting signals. However, only two of these extensions remotely resembled known presequences of hydrogenosomal proteins. The basic amino acid arginine occupies either position −2 or position −3 from the predicted cleavage site in all presequences of putative hydrogenosomal precursor proteins inferred in the *T. vaginalis* genome sequencing project (8). The N-terminal extension of TrxR_92349, though having Arg at a suitable distance (position 12) from the start, possessed no Ser within first 13 amino acids (Fig. 1), and the gene product was not imported into hydrogenosomes (Fig. 2). On the other hand, in spite of its atypical N-terminal extension sequence (Fig. 1), the majority of TrxRh1 clearly localized to hydrogenosomes. This observation was in contrast to the exclusively cytosolic localization of the other two TrxR isoforms with N-terminal extensions relative to TrxRc (Fig. 2). However, TrxRh1 did not localize exclusively to hydrogenosomes, with some protein remaining in the cytosol. Despite the unusual Phe at position 2 of the N terminus instead of the typical Leu or Ser, deletion of the first 10 amino acids after methionine in TrxRh1 showed that sequence to be involved in hydrogenosomal targeting (Fig. 4a). Because of its dual localization, we asked whether recombinant TrxRh1 protein carrying a hydrogenosomal presequence from an exclusively hydrogenosome-localized protein instead of its own presequence would be directed entirely to the organelle. PFO A and SCSα presequences did not target TrxRh1 to hydrogenosomes (Fig. 5a). Moreover, SCSα with its presequence deleted is quantitatively targeted to hydrogenosomes (Fig. 5b). This indicates that the presequences of hydrogenosomal enzymes PFO A and SCSα, though cleaved after protein import (21, 27), are not sufficient, and in the case of SCSα are not necessary, for hydrogenosomal localization of these proteins. Instead, yet-uncharacterized internal targeting signals in these proteins would appear to be involved in their organellar targeting. Similarly, TrxRh2 and TrxRh2-Δ21 both were targeted, at least partially, to the hydrogenosomes as well (Fig. 4b), indicating the presence of an internal, albeit ineffective, targeting signal in this protein, too.

Information about the targeting of proteins to *T. vaginalis* hydrogenosomes is still scarce, with only about a dozen cases reported where the N terminus of the hydrogenosomal protein was compared to the sequence inferred from the mRNA (5). Expression in *Trichomonas* allows us examine targeting and the role of presequences in greater detail. Our results reveal that two previously characterized cleaved presequences, those in PFO A and in SCSα, do not carry all (or perhaps any) information required for targeting and that internal targeting sequences are present in the case of SCSα and in TrxRh2. It is possible that internal targeting signals are common among hydrogenosomal proteins, because rubrerythrin and thiol peroxidase, for instance, also do not contain apparent N-terminal transit peptides (41). An internal targeting signal had previously been proposed for hydrogenosomal membrane protein Hmp31 (14), a homolog of mitochondrial carrier family proteins which possess internal an organelle targeting signal(s) also in mitochondria (44). Similar to SCSα, Hmp31 also contains an N-terminal extension that is processed but not necessary for targeting. In contrast, the unconventional presequence of TrxRh1 is necessary for import (Fig. 4a), as is the N-terminal extension of hydrogenosomal ferredoxin, as shown in in vitro import studies (5). The present results clearly indicate that there are internal signals involved in the import of some hydrogenosomal proteins, but the nature of those signals has yet to be specified.

In that sense, hydrogenosomal import mirrors to some extent the situation in mitochondria. Hydrogenosomes and mitochondria are homologous organelles which descend from the same eubacterial endosymbiont (32, 47), and their membrane targeting and protein import mechanisms are conserved (5, 6,

14, 50). The import of matrix proteins to mitochondria is generally dependent upon cleavable, N-terminal presequences, while the import of inner membrane proteins tends to rely upon multiple internal signals (4, 38, 43), but numerous exceptions regarding the distribution and requirements of targeting signals have been described. Examples are (i) N-terminal presequences that do not act as a targeting signal but facilitate the solubility of preprotein, as it seems to determine the folding state (54); (ii) N-terminal presequences that are not essential for import and assembly of the protein (55); (iii) the coexistence of internal and N-terminal targeting signals, whereby the N-terminal cleavable extension is responsible for protein import (36); and (iv) a C-terminal cleavable mitochondrial targeting signal, as was reported for DNA helicase Hmi1p (29). Though the first three examples represent mitochondrial carrier family proteins which are expected to have multiple internal signals, nonconventional import pathways were also reported for matrix proteins, specifically for the nuclearly encoded mitochondrial transcription factor Mtf1p (2). Its translocation appears to be independent of a cleavable N-terminal presequence, and Mtf1p seems to be capable of using alternative import signals present in different regions of the protein (3).

The present results suggest that protein targeting to hydrogenosomes in some cases involves topogenic signals beyond those provided by N-terminal extensions. Given that two such examples were found among three hydrogenosomal proteins tested here, it is possible that this property is more common among hydrogenosomal proteins than among proteins of typical mitochondria. Despite the common ancestry of mitochondria and hydrogenosomes, some differences in their protein import might be expected. The electrochemical gradient generated by proton pumping in mitochondria that is required for protein translocation by TIM22 and TIM23 complexes (13, 38) is generally thought to be missing in hydrogenosomes, which lack cytochromes and membrane-associated electron transport (35). Although there have been reports that trichomonad hydrogenosomes develop transmembrane potential (22), the mechanisms by which this occurs are still obscure. Import of ferredoxin into isolated trichomonad hydrogenosomes was found to be inhibited by the protonophore *m*-chlorophenylhydrazone, albeit at concentrations roughly 10-fold higher than those that inhibit mitochondrial protein import (5). Trichomonad hydrogenosomes have been shown to efficiently import some proteins of *Giardia* mitosomes that possess N-terminal extensions (12). The findings reported here indicate, however, that N-terminal extensions are not always required for import into *Trichomonas* hydrogenosomes and thus raise new questions concerning the prevalence of this type of import and the mechanisms involved.

Neighbor-Net sequence similarity analysis was conducted, comparing the five TrxRs from *T. vaginalis* with selected bacterial and eukaryotic TrxRs (see Fig. S1 in the supplemental material). All five isoforms clearly cluster together, indicating that they represent paralogs which arose through gene duplication in the *T. vaginalis* lineage. First, all three long versions diverged progressively, including hydrogenosomally localized TrxR_89667. Gene duplication giving rise to the two short paralogs obviously occurred last. Notably, eukaryotic TrxRs do not cluster together. TrxRs from *T. vaginalis* are divergent from and more similar to proteobacterial homologs than those of other eukaryotes. The Neighbor-Net topology indicates interdomain gene transfer, though it is not possible to resolve whether it was horizontal gene transfer or endosymbiotic gene transfer, after the acquisition of mitochondrial predecessor.

Intriguingly, TrxRs in *T. vaginalis*, although all descendants through duplication from a single protein and still highly conserved, are localized to different intracellular compartments, and two of them have developed diverse hydrogenosomal targeting mechanisms. Thus, TrxRs appear to be a suitable system to explore the characteristics of hydrogenosomal protein targeting signals in more detail.

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