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ORIGINAL ARTICLE

The N-Terminal Sequences of Four Major Hydrogenosomal Proteins Are Not Essential for Import into Hydrogenosomes of *Trichomonas vaginalis*

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ABSTRACT

The human pathogen *Trichomonas vaginalis* harbors hydrogenosomes, organelles of mitochondrial origin that generate ATP through hydrogen-producing fermentations. They contain neither genome nor translation machinery, but approximately 500 proteins that are imported from the cytosol. In contrast to well-studied organelles like *Saccharomyces* mitochondria, very little is known about how proteins are transported across the two membranes enclosing the hydrogenosomal matrix. Recent studies indicate that—in addition to N-terminal transit peptides—internal targeting signals might be more common in hydrogenosomes than in mitochondria. To further characterize the extent to which N-terminal and internal motifs mediate hydrogenosomal protein targeting, we transfected *Trichomonas* with 24 hemagglutinin (HA) tag fusion constructs, encompassing 13 different hydrogenosomal and cytosolic proteins of the parasite. Hydrogenosomal targeting of these proteins was analyzed by subcellular fractionation and independently by immunofluorescent localization. The investigated proteins include some of the most abundant hydrogenosomal proteins, such as pyruvate ferredoxin oxidoreductase (PFO), which possesses an amino-terminal targeting signal that is processed on import into hydrogenosomes, but is shown here not to be required for import into hydrogenosomes. Our results demonstrate that the deletion of N-terminal signals of hydrogenosomal precursors generally has little, if any, influence upon import into hydrogenosomes. Although the necessary and sufficient signals for hydrogenosomal import recognition appear complex, targeting to the organelle is still highly specific, as demonstrated by the finding that six HA-tagged glycolytic enzymes, highly expressed under the same promoter as other constructs studied here, localized exclusively to the cytosol and did not associate with hydrogenosomes.

A broad spectrum of eukaryotes from diverse phylogenetic lineages inhabit oxygen-poor or anoxic environments (van der Giezen 2009; Müller et al. 2012; Stoeck et al. 2010). The mitochondria of eukaryotic anaerobes generate ATP through pyruvate breakdown using terminal electron acceptors other than oxygen, and two basic types can be distinguished: those that generate ATP with the help of membrane-associated proton pumping—anaerobic mitochondria—and those that generate ATP via substrate level phosphorylation alone, typically with the production of molecular hydrogen as a major end product—hydrogenosomes (Tielens et al. 2002). Mitochondria, anaerobic mitochondria, hydrogenosomes, and their more reduced forms, mitosomes, are specialized forms of one and the same mitochondrial endosymbiont (Embley and Martin 2006; Embley et al. 2003). Various intermediate forms thereof, including hydrogen-producing mitochondria (Boxma et al. 2005) and facultatively anaerobic mitochondria are known (Ginger et al. 2010). Most hydrogenosomes have entirely relinquished their own DNA (Clemens and Johnson 2000; van der Giezen 2009), in line with their fermentative nature and the observation that membrane-associated electron transport is the main selective force behind organelle genome retention (Allen 2003)—once membrane-associated electron transport is relinquished, the genome is as well. Hydrogenosomes nonetheless harbor several hundred proteins that are synthesized on cytosolic ribosomes and imported into the organelle (Mentel et al. 2008; Schneider et al. 2011).

Many components of the mitochondrial protein targeting machinery are conserved in *Trichomonas* and precursor proteins are thought to be translocated across the two hydrogenosomal membranes by components homologous to those found in the membranes of mitochondria: translocons of the outer and inner mitochondrial membrane, TOMs and TIMs, respectively. These conserved components include *Tv*Tom40, *Tv*Sam50 (sorting and assembly machinery), *Tv*Tim17, *Tv*Tim44, and *Tv*Tim18/Pam16 (Carlton et al. 2007; Dolezal et al. 2006; Rada et al. 2011; Shiflett and Johnson 2010). Still, the import machinery in *T. vaginalis* is thought to be less complex than in yeast, as for example the outer membrane proteins Tom70, Tom20, and Tom22 and the inner membrane proteins Tim54, Tim50, and Tim21 seem to be lacking (Rada et al. 2011; Shiflett and Johnson 2010). An electrochemical gradient, as necessary for protein translocation through mitochondrial Tim22 and Tim23, seems absent in *Trichomonas* hydrogenosomes, which do not possess cytochromes and a membrane-associated electron transport (Dolezal et al. 2005; Müller 1993; Neupert and Herrmann 2007). Isolated reports, however, suggest the opposite and that *Trichomonas* hydrogenosomes do develop a transmembrane potential (Humphreys et al. 1998), but the mechanism of how this might occur remains obscure (Mentel et al. 2008). The import into hydrogenosomes is, as in typical higher eukaryotic mitochondria, temperature- and ATP-concentration-dependent (Bradley et al. 1997). In *T. vaginalis*, duplications of genomic fragments are very common, such that the majority of genes are present in multiple, but not always identical copies (Carlton et al. 2007), further complicating matters. For *Tv*Tom40 there are six copies, for *Tv*Tim17 five, and for *Tv*Tim9/10 at least two (Rada et al. 2011). Expression evidence for each of them at TrichDB (Aurrecoechea et al. 2009) suggests that every single copy is in fact integrated into the hydrogenosomal membranes after their translation.

The hydrogenosomal import components are predicted to import about 500 proteins, an estimated half of which carries a short N-terminal extension (Burstein et al. 2012; Carlton et al. 2007; Dyall and Johnson 2000; Schneider et al. 2011), we shall refer to these as the hydrogenosomal targeting sequence (HTS) in this article. The HTS varies in length from only four amino acids to a maximum of around 24 and a median length of 12 (Fig. 1A). Apart from the very N-terminal amino acids and the cleavage motif, there is no recognizable primary sequence conservation. Experimental evidence exists that the HTS is cleaved by the hydrogenosomal processing peptidase, a homolog of the yeast mitochondrial processing peptidase (Brown et al. 2007; Smid et al. 2008). Nevertheless, detailed functional information about the hydrogenosomal targeting signals of *T. vaginalis* matrix proteins is still scarce and, to complicate the matter, partly ambiguous. In an early study, Bradley et al. (1997) reported the importance of cleavable N-terminal signals based on in vitro import analysis and more recent work confirmed those findings for a thioredoxin reductase isoform (*Tv*TrxRh1; TVAG_281360), using in vivo localization experiments: the deletion of the HTS (MFSIIFFSRF) aborts import and its absence is not compensated by an internal targeting signal, but evidence for the existence of internal targeting information was reported as well (Mentel et al. 2008). The alpha subunit of *Trichomonas* succinyl coenzyme A synthetase (*Tv*SCSα1; TVAG_165340) and yet another isoform of the thioredoxin reductase (*Tv*TrxRh2, TVAG_125360) were imported despite their N-terminal region being deleted (MLSSSFERN and MSGDIDWTKAETVDIAIIGSGP, respectively) (Mentel et al. 2008). In addition, using a supported vector machine trained on hydrogenosomal proteins to screen for patterns among 57 individual features, several hydrogenosomal proteins lacking a canonical targeting signal were identified and the localization of two more was verified (Burstein et al. 2012).

Taken together the current data suggest that protein import into *T. vaginalis* hydrogenosomes is not strictly dependent upon cleavable N-terminal extensions, at least not for all imported proteins, but to what extent internal signals might also be sufficient to direct proteins to the organelle is so far unresolved. Here, we investigate whether the N-terminal HTSs are required for hydrogenosomal import in proteins that possess them and present new evidence indicating that internal import targeting signals are widespread among *Trichomonas* hydrogenosomal proteins.

MATERIALS AND METHODS

Cultivation and cloning

Trichomonas vaginalis strain T1 (J.-H. Tai, Institute of Biomedical Sciences, Taipei, Taiwan) was grown in TYM medium at 37°C as described previously (Gorrell et al. 1984). *Escherichia coli* DH5α was used for standard gene cloning and grown at 37°C after transformation in LB medium supplemented with the appropriate antibiotic. Open reading frames were amplified by PCR using genomic DNA of *T. vaginalis* T1 as a template and sequence-specific primers for each gene (Table S1). For the deletion of 5′ regions encoding the HTS, genes were amplified from cloned full-length genes. Forward and reverse primers contained either *Nde*I and *Bam*HI restriction sites or Gateway recombination sites (Katzen 2007), respectively (Table S1), for cloning the genes into the pTagVag2 expression vector (Hrdy et al. 2004). All pTagVag2 expressed gene products were thus marked with a C-terminal di-hemagglutinin tag (HA-tag) followed by an in frame stop codon. Plasmids were verified by sequencing before transfection. *Trichomonas vaginalis* T1 cells were electroporated as described before in the presence of 50 μg plasmid DNA (Delgadillo et al. 1997; Land et al. 2004) and selected in TYM medium containing 100 μg/ml G418 (Roth, Karlsruhe, Germany).

Cell fractionation and localization analysis

Isolation of hydrogenosomes was based on previous protocols (Bradley et al. 1997; Pütz et al. 2005) with slight modifications. Cells were lysed by grinding them in a

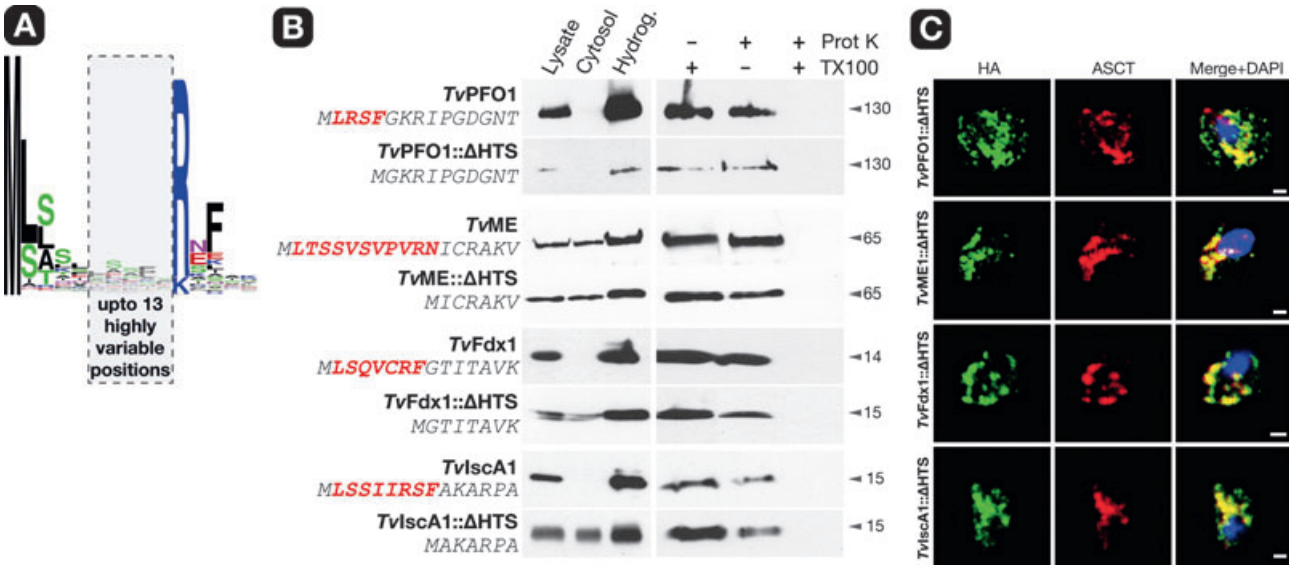

Fig. 1. Deletion of the N-terminal motif does not abort hydrogenosomal matrix targeting. (A) Sequence logo based on 235 putative hydrogenosomal targeting signals extracted from the genome of *Trichomonas vaginalis*. Apart from a short N-terminal region and a stretch preceding the cleavage site, no primary sequence is conserved. (B) Four highly abundant hydrogenosomal proteins (pyruvate ferredoxin oxidoreductase, *Tv*PFO1; malic enzyme, *Tv*ME1; ferredoxin, *Tv*Fdx1; iron–sulfur cluster assembly protein, *Tv*IscA1) are translocated into the hydrogenosomal matrix with and without their N-terminal hydrogenosomal targeting sequence (HTS). Presence (+) or absence (–) of Proteinase K (Prot K) and Triton X-100 (TX100) are indicated above the lanes, in which the protease protection assay is shown. All proteins were C-terminally HA-tagged. The N-terminal amino acid sequences are shown below the constructs name in italic, including in bold red the part of the HTS that was deleted. The approximate molecular weight of the constructs is indicated in kilodaltons on the right. (C) Immunofluorescent co-localization of the four constructs, for which the HTS was deleted, together with the hydrogenosomal marker enzyme acetate:succinate CoA-transferase (ASCT), confirming the western blot results of the subcellular fractions. Scale bar: 2 μm.

mortar in the presence of glass beads (ø 0.4–0.6 μm) on ice. Glass beads were then removed by centrifugation (755*g*, 10 min at 4°C) and whole cell lysate was collected from the supernatant. Cytosolic fraction was obtained from the supernatant after centrifugation at 7,500*g* for 10 min and 4°C. Hydrogenosomes were obtained by isopycnic centrifugation with 45% (v/v) Percoll. For separation of hydrogenosomal matrix and hydrogenosomal membranes, intact organelles were treated with 100 mM Na_2CO_3 and incubated on ice for 45 min. Membranes were then pelleted by ultracentrifugation (208,000*g*, 1 h at 4°C). The pellet was washed once with ddH_2O and then resuspended in 100 μl ddH_2O. Matrix proteins (supernatant) were precipitated with acetone/TCA (7.5:1) and resuspended in 70 μl resuspension buffer (7 M urea; 2 M thiourea; 4% [w/v] CHAPS) and 10 μl concentrated Tris to neutralize the pH. Protein concentrations were determined using a Bradford assay kit (Bio-Rad). Samples (20–50 μg of protein, in a few cases 500–1,000 μg, as stated in the text) for western blot analysis were run on 12–15% SDS-PAGEs and blotted onto nitrocellulose membranes (Hybond-C Extra, GE Healthcare, Freiburg, Germany). Blots were blocked for an hour in TBS (20 mM Tris-HCl pH 7.4; 150 mM NaCl) containing 5% (w/v) dried milk powder and then incubated for a minimum of 1 h at RT, or overnight at 4°C, with 1:5,000–1:20,000 in blocking solution-diluted mouse anti-HA monoclonal antibody (Sigma, Steinheim, Germany). After three washes for 10 min in TBS, the membrane was incubated with the secondary and horseradish peroxidase-conjugated antibody (ImmunoPure, Pierce, Fisher Scientific, Schwerte, Germany) diluted at 1:10,000–1:20,000 in blocking solution. After washing like before, signals were visualized using 3 ml solution A (1.25 mM luminol [Sigma] in 0.1 M Tris-HCl pH 8.6), 300 μl solution B (6 mM para-hydroxycoumaric acid [Sigma] in DMSO), 0.9 μl 30% (v/v) H_2O_2 and Lumi-Film chemiluminescent detection films (Roche, Mannheim, Germany).

As a contamination control of the cytosolic fraction, western blots using antibodies against hydrogenosomal marker proteins (acetate:succinate CoA-transferase ASCT or succinyl CoA synthetase alpha subunit SCSα) were performed. For this purpose, membranes were stripped by incubation in mild stripping buffer (200 mM glycine; 1% [w/v] SDS; 1% [v/v] Tween 20; pH 2.2) for 10 min, followed by two washing steps, each with PBS (0.8% [w/v] NaCl; 0.02% [w/v] KCl; 0.144% [w/v] Na_2HPO_4; 0.024% [w/v] KH_2PO_4) for 10 min and TBS-T (20 mM Tris-HCl pH 7.4; 150 mM NaCl; 0.1% [v/v] Tween 20) for 5 min. Then the western blot was performed as described above.

Protease protection assays were performed with freshly isolated hydrogenosomes. The organelles were washed twice in 1 ml SM buffer (10 mM MOPS/KOH pH 8.0; 250 mM sucrose; 10 mM EDTA) with centrifugations at 2,500*g* for 10 min in-between. The samples were then

resuspended to a concentration of 2 μg/μl hydrogenosomes in the same buffer. Triton X-100 was added to the half of the sample. Both samples were incubated for 30 min at 37°C. After incubation both samples were partitioned and individually 50 and 100 μg/ml Proteinase K (Roth Chemicals) added. For a negative control to one sample no Proteinase K was added. All samples were again incubated for 30 min at 37°C. The assay was terminated by adding 3/4 of volume of acetone/TCA (7.5:1) and incubation over night at −20°C. Samples were then centrifuged at 16,060*g* for 10 min and washed three times with acetone and incubated for 5–20 min at RT and 500 rpm. Proteins were resuspended in resuspension buffer (7 M urea; 2 M thiourea; 4% [w/v] CHAPS) on a thermal shaker for 30 min at 30°C and 600 rpm. Concentrated Tris was added to adjust the samples to a neutral pH before SDS-PAGE separation.

Expressed HA-tagged proteins and ASCT (hydrogenosomal marker) were visualized in fixed *T. vaginalis* cells with mouse antihemagglutinin monoclonal antibody (Sigma-Aldrich, Germany) and rabbit anti-ASCT polyclonal antibody (van Grinsven et al. 2008) as primary antibodies and secondary Alexa-Fluor-488 donkey anti-mouse and Alexa-Fluor-633 donkey anti-rabbit antibodies (Invitrogen, Karlsruhe, Germany). Images were obtained using a LSM 510 Meta confocal laser scanning microscope (Zeiss, Germany). Cells from a logarithmic phase *T. vaginalis* culture were fixed in an anaerobic chamber on silane-coated microscopic slides (Electron Microscopy Sciences, Hatfield) in two subsequent steps by methanol (5 min) and acetone (5 min) at −20°C and then treated with 0.25% gelatin/BSA in PBS (8% [w/v] NaCl, 0.2% [w/v] KCl, 1.44% [w/v] Na_2HPO_4, 0.24% [w/v] KH_2PO_4, pH 7.4) for 1 h at room temperature. The slides were then incubated with both primary antibodies (diluted 1:500 in PBS plus 0.25% gelatin/BSA) for 1 h at room temperature. After three 10 min washes in PBS, the slides were incubated with secondary antibodies (diluted 1:1,000 in PBS with 0.25% gelatin and 0.25% BSA) for 1 h at room temperature in the dark. After washing as described above, the slides were mounted in Fluoroshield with DAPI (Sigma-Aldrich, Germany).

RESULTS

Highly abundant hydrogenosomal proteins do not require their HTS

To home in on the role of N-terminal signals, we first investigated the targeting of four abundant hydrogenosomal proteins encoding a typical HTS: pyruvate ferredoxin oxidoreductase (*Tv*PFO1; TVAG_198110), malic enzyme (TVAG_267870), ferredoxin (*Tv*Fdx1; TVAG_003900), and an iron-sulfur cluster assembly protein (*Tv*IscA1; TVAG_456770). Subcellular fractionation and C-terminal HA-tagging showed that, as expected, all four full-length proteins were translocated into the hydrogenosomal matrix, as revealed by the protease protection assay (Fig. 1B). Next we removed the N-terminal presequences based on their known processing sites and compared their localization with that of the full-length proteins. All four HTS-lacking constructs (*Tv*PFO1::ΔHTS, *Tv*ME1::ΔHTS, *Tv*Fdx1::ΔHTS and *Tv*IscA1::ΔHTS) were not only associated with the hydrogenosomal fraction but also imported into the matrix. In addition, a minor signal was then also detectable in the cytosolic fraction of *Tv*Fdx1::ΔHTS and *Tv*IscA1::ΔHTS. Western blot signals were only detectable when no Triton X-100 was added to lyse the isolated organelles, making the imported protein accessible to the protease (Fig. 1B). The hydrogenosomal localization of *Tv*PFO1::ΔHTS, *Tv*ME1::ΔHTS, *Tv*Fdx1::ΔHTS, and *Tv*IscA1::ΔHTS was furthermore verified using immunofluorescent co-localization with acetate:succinate CoA-transferase (ASCT) as a hydrogenosomal marker enzyme (Fig. 1C).

Although using cell fractionation as a method to determine subcellular localization of hydrogenosomal proteins is common practice (Burstein et al. 2012; van Grinsven et al. 2008; Mentel et al. 2008; Pütz et al. 2006), we wanted to exclude the unlikely possibility of a systematic error regarding the import assay results, in particular we wanted to exclude the possibility that highly expressed proteins might localize nonspecifically to hydrogenosomes. For that, we generated six different glycolytic enzymes C-terminal HA-tagged constructs under the control of the same strong *Tv*SCSα1 promoter used for all other constructs, and determined their localization via cell fractionation: glucokinase (*Tv*GK; TVAG_023840), glucose-6-phosphate isomerase (*Tv*GPI; TVAG_061930), fructose-1,6-bisphosphate aldolase (*Tv*FBA; TVAG_043170), glyceraldehyde-3-phosphate dehydrogenase (*Tv*GAPDH; TVAG_475220), phosphoglycerate kinase (*Tv*PGK; TVAG_268050), and enolase (*Tv*ENO; TVAG_043500). These are well-characterized cytosolic marker proteins for *Trichomonas vaginalis* (Müller 2003). The proteins were cloned using the same vectors and methods we used for hydrogenosomal proteins. The glycolytic enzymes were heavily expressed, as expected, but no signal was detected within the hydrogenosomal fractions (Fig. 2). This result rules out localization artifacts stemming from *Tv*SCSα1 promoter-dependent overexpression or possible C-terminal HA-tagging effects and underpins further the general utility of the *Tv*SCSα1 promoter/HA-tag import assay. We additionally compared the expression and localization of the endogenous copy of *Tv*ASCT1 (TVAG_395550) using a specific antibody (van Grinsven et al. 2008), with that of a tagged version expressed under the *Tv*SCSα1 promoter. Again no obvious difference was observed (Fig. 2).

Individual fragments of *Tv*SCSα1 and *Tv*TrxRh2 target to the matrix

For proteins in which the HTS has a negligible effect upon hydrogenosomal targeting, the information specifying hydrogenosomal import must be located within the mature subunit. To verify and better understand internal targeting information, we divided two different, highly abundant hydrogenosomal proteins into three separate sections of

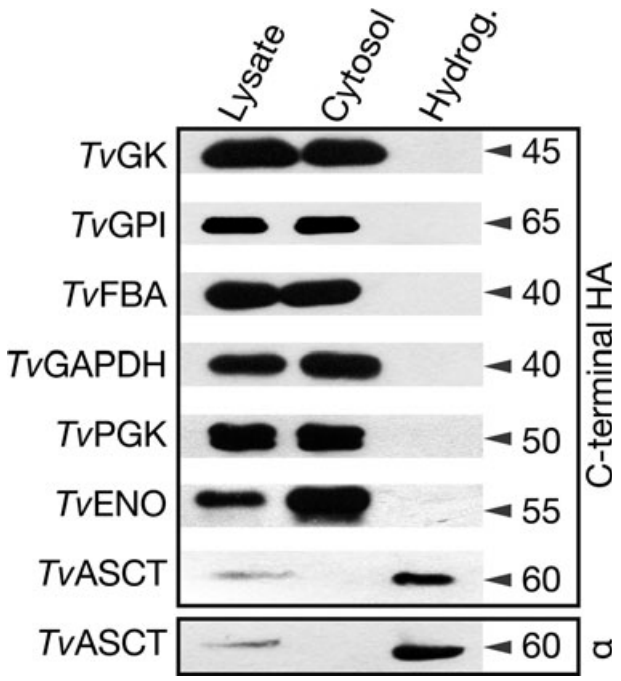

Fig. 2. Localization of glycolytic enzymes in *Trichomonas vaginalis*. As controls for cytosolic localization the coding sequences for glucokinase (GK), glucose-6-phosphate isomerase (GPI), fructose-1,6-bisphosphate aldolase (FBA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), and enolase (ENO) were expressed with HA-tags under the control of the *Tv*SCSα1 promoter. In addition, we compared the expression of acetate:succinate CoA-transferase under the endogenous promoter (α, antibody against ASCT) and under that of the *Tv*SCSα1 when HA-tagged. The approximate molecular weight of the constructs is indicated in kilodaltons on the right.

almost identical length. One of the so subdivided hydrogenosomal proteins does encode an HTS (*Tv*SCSα1; Fig. 3) while one does not (*Tv*TrxRh2; Fig. S1). The three blocks of *Tv*SCSα1 spanned amino acids 1 to 101 (*Tv*SCSα1-1), 102 to 201 (*Tv*SCSα1-2), and 202 to 301 (*Tv*SCSα1-3) of the *Tv*SCSα1 sequence lacking the HTS. *Tv*SCSα1-1 and *Tv*SCSα1-2 were both imported into the hydrogenosomal matrix, but approximately the same amount of protein was detected in the cytosolic fraction, indicating that only a portion of the HA-tagged protein was imported (Fig. 3). The cytosolic signal is not just caused by the contamination of lysed hydrogenosomes, as shown by stripping and reblotting the membrane with a hydrogenosomal marker (αASCT) as a control, which revealed the cytosolic fraction to be free from hydrogenosomal contamination (Fig. S2). Notably, a clear double band of almost equal intensity was present in the hydrogenosomal fraction and to a minor degree in the total cell extract (Fig. 3A) . The additional band corresponds to an approximately 1 kilodalton heavier protein and does not represent a degradation product. The faster migrating band conforms to the predicted size of the constructs. Again we treated the isolated and intact organelles with proteinase K, which showed the faster migrating bands to be protected from the protease and giving evidence for their translocation across the hydrogenosomal membranes. The slower migrating band, however, showed reduced intensity after adding Triton X-100 or Proteinase K individually. The hydrogenosomal localization of *Tv*SCSα1, *Tv*SCSα1::ΔHTS and the first two blocks of *Tv*SCSα1 were furthermore verified by immunofluorescent co-localization with ASCT (Fig. 3B). The C-terminal block *Tv*SCSα1-3 was not expressed at a satisfactory level—even after multiple transfections—to allow a reliable localization prediction. From the second transfection round, 10 times the protein amount (500 μg) compared to block 1 and 2, had to be loaded to obtain any signal. A marginal amount of *Tv*SCSα1-3 was then detected in the hydrogenosomal fraction (Fig. 3A). This signal, however, was degraded when adding Proteinase K and no Triton X-100 in the protease protection assay (Fig. 3A), suggesting only association with the hydrogenosomes, but no true import.

Using *Tv*TrxRh2, we performed an experiment identical to the one for *Tv*SCSα1, because Mentel et al. (2008) had shown *Tv*TrxRh2 to lack a canonical HTS and to be imported into hydrogenosomes without its N-terminal domain. We split the *Tv*TrxRh2 sequence into three blocks ranging from amino acid 1 to 116, 117 to 229, and 230 to 311. As with *Tv*SCSα1, the first two blocks were incompletely imported, also with an additional slower migrating band within the hydrogenosomal fraction. The third block also showed hydrogenosomal association, but no obvious double band (Fig. S1). When we performed protease protection assays to distinguish between hydrogenosome import and association only, no signals were detectable, although all cell lines showed no altered growth despite the added selection drug. This problem remained even after two re-transfections, with highly variable and nonstable detection levels regarding the individual constructs. However, because of the presence of the additional slower migration band in the hydrogenosomal fraction of at least block 1 and 2, we observed a targeting behavior similar to that of the *Tv*SCSα blocks.

DISCUSSION

The first characterized gene encoding a hydrogenosomal protein (*Tv*Fdx1) contained a short N-terminal presequence, which was found absent from the mature protein (Johnson et al. 1990; Smid et al. 2008), highly reminiscent of the situation in mitochondria known at the time. Subsequent studies showed similar short presequences in many hydrogenosomal proteins in *Trichomonas vaginalis* (Hrdy and Müller 1995a,b; Lahti et al. 1992, 1994; Lange et al. 1994; Mentel et al. 2008). Although several experiments have provided evidence for the processing of the HTS (Bradley et al. 1997; Hrdy and Müller 1995a; Lange et al. 1994; Sutak et al. 2004), the functional role of the HTS in the actual targeting process, when the protein passes the two hydrogenosomal membranes, has not been well-studied (van der Giezen 2009).

Ultimately, the only HTS we know that obeys the classical assumption that import into hydrogenosomes of *Trichomonas* depends on N-terminal targeting peptides is

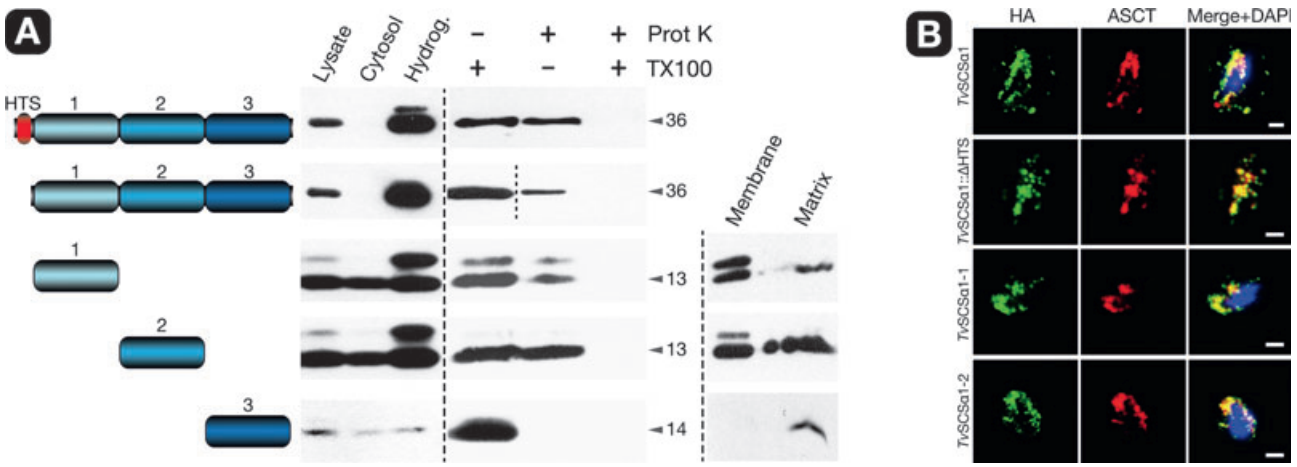

Fig. 3. Individual domains of *Tv*SCSα1 are sufficient for correct matrix localization. (A) While blocks 1 and 2 of *Tv*SCSα1 are truly imported into the organelles, as they are protease (Prot K) protected, the third block seems only associated with the hydrogenosomes and is digested even in the absence of Triton X-100 (TX100). For constructs showing a clear double band, the hydrogenosomes were furthermore separated into a membrane and matrix fraction, revealing the slower migrating band to be associated with the membranes. Dashed lines indicate that samples did not run on the same SDS-PAGE. Fifty micrograms of protein extract was loaded, except for block 3, in which 500 μg were loaded. For further details refer to Fig. 1 and the text. (B) Immunofluorescent co-localization of ASCT with the full-length *Tv*SCSα1, with a deleted HTS (*Tv*SCSα1::ΔHTS), and the first block of *Tv*SCSα1 (amino acids 1 to 101 of *Tv*SCSα1::ΔHTS) and the second block of *Tv*SCSα1 (amino acids 102 to 201 *Tv*SCSα1::ΔHTS). Scale bar: 2 μm.

that of *Tv*TrxRh1 (Mentel et al. 2008). In two other cases tested previously, the importance of the HTS seems negligible, as recently shown for *Tv*SCSα1 (Mentel et al. 2008) and by Dyall and colleagues (Dyall et al. 2000) for *Tv*Hmp31 (TVAG_237680). In both the cases, the deletion of the HTS did not affect correct hydrogenosomal localization. The present study expands the list of functionally characterized HTSs by four biochemically important and highly abundant hydrogenosomal proteins, namely PFO, malic enzyme, ferredoxin, and IscA. These proteins do not require their N-terminal HTS for correct targeting in vivo. For ferredoxin this stands in contrast to the previous, carefully performed in vitro experiments by Bradley and colleagues (Bradley et al. 1997), who reported that, similar to mitochondrial protein import, the presequence of ferredoxin was essential for translocation. Our results show, however, that in vivo this is not the case and that ferredoxin is imported with and without the N-terminal targeting signal. This might be a strain-specific occurrence (C1 vs. T1) or due to the generally very different methods used to analyze the targeting properties of the different constructs (e.g. HIS vs. HA-tag; in vivo vs. in vitro). Possible is furthermore the need for cytosolic factors aiding import, which are lacking in the in vitro experiment, and whose absence can apparently be compensated in vitro by the presence of an HTS, or the physiological state of the hydrogenosomes in vivo versus in vitro might influence ferredoxin import. Although the presequences are processed, our results clearly show that for the proteins investigated, the HTS is not required for correct subcellular localization. Presequence processing inside the hydrogenosomal matrix might, however, be required for the mature subunits to correctly carry out their function.

We noticed that in some cases a recognizable amount of the tagged protein was found in the cytosolic fraction after, for example the deletion of the N-terminus (Fig. 1B for *Tv*Fdx1::ΔHTS and *Tv*IscA1::ΔHTS) and our controls indicate this not to be a contamination artifact (Fig. S3), which suggests that in a few cases the HTS augments the import process. This observation might be connected to the (currently obscure) reasons why, for the fragmented sequences of *Tv*SCSα1 and *Tv*TrxRh2, a substantial amount of protein was also identified in the cytosolic fraction, which might indicate incomplete import. The interplay of individual motifs, i.e. of the HTS and an internal motif, might specify correct and outright import. An overexpression artifact is unlikely in that case, as the comparison of the endogenously expressed *Tv*ASCT1 with that under of the control of *Tv*SCSα1 promoter showed no observable difference (Fig. 2), and we furthermore observed an equal amount of protein in the cytosolic and hydrogenosomal fraction in only a few cases.

At least four additional matrix proteins have been shown to lack an HTS, but to be hydrogenosomal: *Tv*TrxRh2, TVAG_479680, TVAG_221830, and a rubrerythrin isoform (TVAG_064490) (Burstein et al. 2012; Mentel et al. 2008; Pütz et al. 2005). Proteins whose localization has not been verified experimentally, but that are thought to be hydrogenosomal and lack obvious N-terminal targeting sequences furthermore include other rubrerythrin isoforms (TVAG_178060, TVAG_378980) and two thiol peroxidases (TVAG_055200, TVAG_165690). Together with the present, albeit limited sample, of experimentally analyzed proteins, it appears that internal targeting motifs are not an exception, but might be rather common. This would tend to parallel the situation in yeast, where up to 30% of

mitochondrial proteins have a variety of different internal import signals (Chacinska et al. 2009; Vögtle et al. 2009), but the extent for hydrogenosomes is not yet known. Furthermore, when present, the HTS appears to be of far lesser importance than the mitochondrial transit peptide in yeast (Chacinska et al. 2009). Indeed, for two cases tested here (*Tv*SCSα1 and *Tv*TrxRh2) we could verify the existence of internal targeting motifs within the mature part of the proteins. Surprisingly, these were not limited to a single unique region, but spanned a minimum of two regions (Fig. 3, S1). Due to the very low expression of the third block of the *Tv*SCSα1 and the amount of protein required to detect any signal, this particular result needs to be interpreted with caution. The little bit of signal we detect in the matrix, but not the protease protection assay, might be an artifact or be caused through concentrating the fraction through precipitation—a methodical requirement. That six glycolytic enzymes tagged with HA strictly remained in the cytosol (Fig. 2) indicates that the presence of the HA-tag, and the expression under the control of a hydrogenosomal protein promoter, does not predispose proteins to hydrogenosomal import. It seems unlikely that the internal motifs are recognized via their primary sequence.

Western blot analysis of a few constructs revealed an additional and slower migrating band (Fig. 3, S1, S4). The shift corresponding to approximately 1 kilodalton was predominantly observed within the hydrogenosomal fraction and especially when the endogenous protein sequence altered in some way, i.e. when the HTS was deleted or especially when only a segment of a protein was analyzed for its targeting properties. This modification seems associated with the hydrogenosomal membranes, as no slower migrating protein was detected within the matrix fraction (Fig. 3). The slower migrating band furthermore seems Proteinase K accessible, suggesting that either (i) some protease can penetrate the outer hydrogenosomal membrane or (ii) that membrane is partially lost during organelle isolation or (iii) these constructs are partially inserted into the outer hydrogenosomal membrane. This band could, in principle, represent an import intermediate, but it could also be spurious. However, it was observed sufficiently often to warrant further investigation. Using liquid chromatography-mass spectrometry we attempted to determine of what nature the modification might be. Multiple attempts only retrieved unmodified peptides of the target protein, which did not cover the entire protein sequence though. Further analysis is required to resolve this issue, as the nature of the size shift might hold an important clue for unraveling hydrogenosomal protein recognition and import.

The size shift observed does not correspond to any common posttranslational modifications, such as prenylation, palmitoylation, or ubiquitination. It might relate to the molecular mechanism behind the translocation process and the recognition of precursor proteins lacking canonical N-terminal targeting sequences, or it might relate to a tag marking improperly or incompletely imported proteins for the degradation pathways. The latter seems unlikely, however, as one would predict the amount of signal to decrease due to degradation, which is not the case.

Intriguingly this shift was sometimes also observed for the endogenous unmodified proteins *Tv*ASCT1 (Fig. S2, only in the membrane fraction) and *Tv*SCSα1 (Fig. S1, control for block 3 of *Tv*TrxRh2; Fig. S3 control for malic enzyme), when using specific antibodies against the proteins. Hence, this demonstrates the shift not be an overexpression artifact or somehow be caused by the HA-tagging, but rather to apparently naturally occur to proteins being translocated into the hydrogenosomes of *Trichomonas*. Whether this truly is associated with an import-related step one can only speculate and the true reason remains obscure.

The common ancestry of hydrogenosomes with mitochondria and mitosomes is reflected by some of the characteristics the targeting shares, apart from the import protein components involved. *Trichomonas* generally exhibits short HTSs, on average less than ten amino acids long and as short as four as in the case of *Tv*PFO1 and as summarized above many lack a recognizable N-terminal motif a priori. The same seems true for *Giardia intestinalis*. Mitosomal targeting signals are also relatively short or even completely absent (Burri and Keeling 2007; Dolezal et al. 2005; Regoes et al. 2005). Whereas some N-terminal motifs of mitosomal proteins of *G. intestinalis* seem essential, others do not (Dolezal et al. 2005; Regoes et al. 2005). Furthermore, in the microsporidian parasite *Encephalitozoon cuniculi* those mitosomal proteins, which have been localized to the organelle, lack N-terminal motifs, too (Goldberg et al. 2008; Tsaousis et al. 2008). Altogether this suggest that with the evolutionary reduction of the organelle, the N-terminal motifs are reduced as well, making internal targeting motifs a common feature of hydrogenosomal and mitosomal proteins.

Only about half of the estimated hydrogenosomal proteins of *Trichomonas* possess a recognizable N-terminal HTS. A large amount of yeast mitochondrial proteins also do not use cleavable N-terminal motifs, albeit most of them are membrane proteins or proteins of the intermembrane space (Chacinska et al. 2009). Our results demonstrate, however, that in *Trichomonas* the combination of different targeting signals within one protein is frequent, and the precursors do not solely depend on the more canonical N-terminal motifs, even if they are present. The recognition versatility of *T. vaginalis* precursor proteins that are designated for hydrogenosomal import presents an important context for understanding the mechanisms behind the molecular recognition processes that specify targeting to the organelle.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. As for individual domains of *Tv*SCSα1 (Fig. 3), domains of *Tv*TrxRh2 are also sufficient for hydrogenosome targeting. Blocks 1 and 2 show a similar pattern as the individual blocks 1 and 2 of *Tv*SCSα1 (Fig. 3), but no protein was detectable for block 3. A quantity of 50 μg of protein extract was loaded, except for block 3, in which 500 μg were loaded. For the control westerns, the original blot was stripped and reprobed with anti-SCSα. Blots for which we repeatedly detected no signals are marked with n.d. For further details refer to Fig. 3.

Fig. S2. Controls for Fig. 3. The original blots were stripped and reprobed with the ASCT antibody.

Fig. S3. Controls for Fig. 1. The original blots were stripped and reprobed with antibodies against the hydrogenosomal marker protein ASCT (except for *Tv*ME and *Tv*ME::ΔHTS, where α-SCS was used for size reasons) to analyze for hydrogenosomal contamination of the cytosolic fraction.

Fig. S4. Western blot image of the three *Tv*SCSα blocks (shown separately in Fig. 3) that were separated together on one SDS-PAGE gel and detected using anti-HA. The image shows the different migration behaviors and predicted sizes are given below the construct names.

Table. S1. Primers used in this study. Whether genes were cloned using standard restriction methods or Gateway® technology (Invitrogen) is apparent from the primers name. Either restriction enzymes (*Nde*I or *Bam*HI) or attachment sites (B1 or B2, B5 or B5r) are given.