Trypanosome Alternative Oxidase Possesses both an N-Terminal and Internal Mitochondrial Targeting Signal

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Recognition of mitochondrial targeting signals (MTS) by receptor translocases of outer and inner membranes of mitochondria is one of the prerequisites for import of nucleus-encoded proteins into this organelle. The MTS for a majority of trypanosomatid mitochondrial proteins have not been well defined. Here we analyzed the targeting signal for trypanosome alternative oxidase (TAO), which functions as the sole terminal oxidase in the infective form of Trypanosoma brucei. Deleting the first 10 of 24 amino acids predicted to be the classical N-terminal MTS of TAO did not affect its import into mitochondria in vitro. Furthermore, ectopically expressed TAO was targeted to mitochondria in both forms of the parasite even after deletion of first 40 amino acid residues. However, deletion of more than 20 amino acid residues from the N terminus reduced the efficiency of import. These data suggest that besides an N-terminal MTS, TAO possesses an internal mitochondrial targeting signal. In addition, both the N-terminal MTS and the mature TAO protein were able to target a cytosolic protein, dihydrofolate reductase (DHFR), to a T. brucei mitochondrion. Further analysis identified a cryptic internal MTS of TAO, located within amino acid residues 115 to 146, which was fully capable of targeting DHFR to mitochondria. The internal signal was more efficient than the N-terminal MTS for import of this heterologous protein. Together, these results show that TAO possesses a cleavable N-terminal MTS as well as an internal MTS and that these signals act together for efficient import of TAO into mitochondria.

Import of nucleus-encoded proteins into mitochondria is critical for mitochondrial function. The import pathways of mitochondrial proteins have been extensively documented in fungi and higher eukaryotes (1, 2) and are beginning to be resolved in trypanosomatids (3-6), which represent a group of the earliest branching eukaryotes (7). This reflects the fact that many of the commonly known components of the mitochondrial protein import machinery are either missing or highly divergent in trypanosomatids (4-6).

For most mitochondrial proteins, their import into mitochondria depends on two major prerequisites: (i) the presence of a mitochondrial targeting signal(s) (MTS) within the proteins and (ii) the presence of specific translocators within the mitochondrial membranes to recognize the targeting signals (8). Essentially, three types of MTS have been found in proteins destined for mitochondria: N-terminal signals, stop-transfer or sorting signals, and internal signals (8). The N-terminal targeting sequence, or presequence, is an amphipathic helix consisting of both hydrophobic and basic amino acid residues. This sequence is cleaved by a mitochondrial processing peptidase (MPP) once the preprotein enters the mitochondrial matrix (9). Another type of MTS consists of two parts. The first part is a canonical presequence followed immediately by a hydrophobic patch large enough to span the membrane. This type of signal is known as the stop-transfer signal or the sorting signal and is found in many inner mitochondrial membrane proteins (1, 8, 9). Nucleus-encoded mitochondrial proteins that do not have an N-terminal targeting signal are imported into mitochondria via internal targeting signals (1, 8, 10). For example, multipass inner membrane proteins such as adenosine nucleotide translocase, phosphate, and other metabolite carriers contain such internal targeting signals (2, 11). The characteristics of these internal targeting signals have not been well defined.

As seen with other eukaryotes, a large number of mitochondrial proteins in kinetoplastid parasites, such as Trypanosoma brucei, are nucleus encoded and therefore need to be imported into mitochondria in order to perform their function (3, 12, 13). Import of these proteins is crucial to the parasite’s survival. Many of these nucleus-encoded proteins are synthesized on cytosolic ribosomes with N-terminal extensions, or presequences. These presequences can be up to 18 to 60 amino acids in length as seen in other eukaryotes (14). However, a number of trypanosomatid mitochondrial proteins possess a presequence that can be as short as 8 amino acid residues (3, 12, 15).

Trypanosome alternative oxidase (TAO) is a nucleus-encoded protein that functions as the sole terminal oxidase in the infective form of T. brucei (16), the causative agent of African trypanosomiasis. TAO is partially embedded in the single leaflet of the inner membrane of the mitochondrial, and both the N and C termini are in the mitochondrial matrix (16-18). TAO possesses a putative N-terminal MTS that contains 24 amino acids as predicted by the Mitoprot program (19). Whether this sequence is required and sufficient for import into T. brucei mitochondrion has not been established. Here we show that in addition to a cleavable canonical N-terminal MTS, TAO possesses one or more internal targeting signals that are functional for import into mitochondria. We identified one such signal that maps within residues 115 to 146 and is more efficient in the import process than the N-terminal signal. When fused to a heterologous protein, DHFR, both signals can drive the import of the cytosolic protein into mitochondria.
**Materials and Methods**

Cells. *T. brucei* 427 cells (procyclic form) were grown in SDM-79 medium containing 10% fetal bovine serum. A *T. brucei* 427 procyclic doubly resistant cell line (TB427 29-13) expressing the tetracycline repressor gene (tetR) and T7RNA polymerase (T7RNAP) (20) was grown in the same medium containing 50 μg/ml hygromycin and 15 μg/ml G418. The bloodstream form of *T. brucei* 427 single-marker (SM) cells (21) expressing the tetracycline repressor and T7 polymerase genes was grown in HMI-9 medium (22) containing 2.5 μg/ml G418. For the measurement of cell growth, the procyclic and bloodstream form cells were inoculated in appropriate medium at cell densities of 2 × 10⁶/ml and 2 × 10⁷/ml, respectively. Cells were harvested at different time points of growth (24 to 96 h), and the cells were counted in a Neubauer hemocytometer.

For a large-scale isolation of the bloodstream form cells, Sprague-Dawley rats were infected with the parasite by intraperitoneal injection (10⁷ cells/100 g body weight). Blood was collected from infected animals by cardiac puncture when the parasitemia level reached about 10⁹/ml, which was approximately 3 to 4 days after infection. The bloodstream form trypanosomes were separated from the blood by diethylaminoethyl (DEAE) cellulose chromatography as described previously (23). All animal procedures were performed according to approved guidelines of the Institutional Animal Care and Use Committee.

**Isolation of mitochondria from *T. brucei* parasites.** Mitochondria were isolated by differential centrifugation after lysis of the parasite via nitrogen cavitation in isotonic buffer as described previously (24). Isolated mitochondria were further purified by resuspension in 50% Percoll and centrifuged at 100,000 × g for 60 min using a linear gradient of 20% to 35% Percoll (25). The isolated mitochondria were stored at a protein concentration of ~10 mg/ml in MOPS (morpholinopropanesulfonic acid)/KOH buffer containing 50% glycerol at −80°C.

**Generation of radiolabeled precursor proteins.** The coding regions for full-length (FL) and mutant TAO were PCR amplified using sequence-specific forward and reverse primers (see Table S1 in the supplemental material) containing HindIII and XhoI restriction sites at their 5' ends, respectively. The cDNA clone for TAO was used as the template. The PCR products were purified, digested with the respective enzymes, and then subcloned into the pGEMZ vector between the BamHI and HindIII sites.

Radioabeled precursor proteins were synthesized in vitro using a coupled transcription-translation rabbit reticulocyte lysate system (TNT®, Promega) according to the manufacturer’s protocol using [35S]-methionine.

**Import of proteins into mitochondria in vitro.** Isolated mitochondria from *T. brucei* were used for in vitro assays of protein import as described previously (26). Briefly, mitochondria (100 μg) were washed with 9 volumes of SME buffer and resuspended in 90 μl of import buffer (250 mM sucrose, 80 mM KCl, 5 mM MgCl₂, 5 mM dithiothreitol, 10 mg/ml fatty acid-free bovine serum albumin, 10 mM MOPS/KOH at pH 7.2, 2 mM ATP, 10 mM creatine phosphate, 0.1 mg/ml creatine kinase, 8 mM potassium ascorbate, 0.2 mM N,N,N',N'-tetramethylphenylenediamine, and 5 mM NADH). The mitochondrial suspension was mixed with ~10 μl of the rabbit reticulocyte TNT mixture containing the radiolabeled precursor protein and incubated at room temperature for up to 20 min. After incubation, mitochondria were washed twice with 500 μl of SME buffer (20 mM MOPS/KOH, pH 7.4, 250 mM sucrose, 2 mM EDTA) to remove excess radiolabeled proteins. Mitochondrial proteins were then separated by SDS-PAGE and transferred onto nitrocellulose membrane. After transfer, the blot was dried at 37°C for 30 min and exposed to an X-ray film (Biomax film; Kodak) for detection of radioactive proteins. For some experiments, the postimport mitochondrial fraction was treated with Na₂CO₃ (0.1 M; pH 11.5) for 30 min at 4°C and then centrifuged at 12,000 × g for 10 min to separate integral membrane and soluble proteins. To test for the requirement of a mitochondrial membrane potential for import of proteins, mitochondria were pretreated with valinomycin (5 μM) and carbonyl cyanide m-chlorophenylhydrazone (CCCP) (50 μM) before radiolabeled precursor proteins were added.

**Immunoprecipitation of TAO and MS analysis.** TAO was immunopurified using a cross-link immunoprecipitation (IP) kit (Thermo Scientific). ImmunoPure Immobilized Protein G Plus slurry (40 μl) was incubated with polyclonal anti-TAO antiserum (500 μl). The antibody and slurry were cross-linked using disuccinimidyl suberate (DSS), after which mitochondrial lysate from both procyclic (2 mg of mitochondrial proteins) and bloodstream (500 μg of mitochondrial proteins) parasites was added to the column and incubated overnight at 4°C. The column was washed, and bound proteins were eluted using elution buffer. Proteins were separated by SDS-PAGE, and the protein band for TAO was detected by the use of an anti-TAO monoclonal antibody. The corresponding protein bands were excised from the Coomassie-stained gel, digested with trypsin, and analyzed by mass spectrometry (MS). The MS/MS spectra were compared to data in the *T. brucei* protein database downloaded from the Gene DB server.

**Generation of plasmid constructs for expression of wild-type and mutant TAO.** For expression of the C-terminal 3×-hemagglutinin (HA) antigen epitope-tagged TAO, the coding region was amplified from a cDNA clone of TAO using sequence-specific forward and reverse primers (see Table S1) for generation of N-terminal deletion constructs (Δ10TAO-3HA, Δ20TAO-3HA, Δ30TAO-3HA, and Δ40TAO-3HA), and the same reverse primer was used for generation of the full-length TAO construct. Digest and purified PCR products were subcloned into a pLEW100-3HA vector (a generous gift from Xiaoming Tu) (27) between the HindIII and Xhol sites. For generation of the TAO-DHFR fusion constructs, FLTAO and TAO fragments (amino acid residues 1 to 30 and 31 to 329 of TAO) were amplified using forward and reverse primers (see Table S1) containing HindIII and BamHI restriction sites at the 5’ ends, respectively. The mouse DHFR open reading frame (ORF) was PCR amplified using pQE16 vector (Qiagen) as the template and the forward and reverse primers (see Table S1) containing BamHI and Xhol restriction sites at the 5’ ends, respectively. PCR products for TAO and DHFR were digested with appropriate restriction enzymes and cloned into pLEW100-3HA vector between the HindIII and Xhol sites. The purified plasmid DNA was linearized by NotI and used for transfection into the procyclic form (TB427 29-13) or bloodstream form (TB427 SM) of *T. brucei* according to standard protocols (20, 21), and the products were selected by phleomycin (2.5 μg/ml) resistance. After transfection, the linearized plasmid was integrated into the ribosomal DNA spacer region in *T. brucei*. Expression of tagged proteins was induced using doxycycline. Various concentrations of doxycycline (0.5 to 5.0 μg/ml) were used to adjust the expression levels of different TAO variants.

**Cell fractionation.** Fractionation of *T. brucei* cells was performed as described previously (28). Briefly, 2 × 10⁸ cells were resuspended in 500 μl of SME buffer (20 mM MOPS/KOH [pH 7.4], 250 mM sucrose, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF]) containing 0.03% digitonin and incubated on ice for 5 min. The cell suspension was then centrifuged for 5 min at 6,800 × g at 4°C. The resultant pellet was considered the crude mitochondrial fraction, and the supernatant contained soluble cytosolic proteins.

**SDS-PAGE and immunoblot analysis.** Total cellular proteins and proteins from isolated mitochondria were analyzed on SDS-PAGE (12%) and transferred to nitrocellulose membranes as described previously (24, 26). Blots were treated with polyclonal antibodies against the *T. brucei* voltage-dependent anion channel (VDAC) (29), *T. brucei* protein phosphatase 5 (TbPP5) (30), and *T. brucei* mitochondrial RNA-binding protein (RBP16) (31) and with monoclonal antibodies for HA (abcam) and TAO (32). Appropriate secondary antibodies were used, and blots were developed using an enhanced chemiluminescence (ECL) detection system (Pierce).

**MitoTracker staining.** MitoTracker Red CMXROS (Invitrogen) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 mM and added to a final concentration of 0.5 μM for procyclic form and 0.05 μM for bloodstream form.
for bloodstream form *T. brucei* (24). The cell suspension was incubated at the respective growth temperatures for 10 min. Cells were washed and incubated in fresh culture medium appropriate for the procyclic form and the bloodstream form for an additional 30 min under normal growth conditions. Cells were collected by centrifugation and immediately used for immunostaining.

**Immunofluorescence microscopy.** *T. brucei* cells (4 × 10^6 to 5 × 10^6) were evenly spread over poly-L-lysine (100 μg/ml in H2O)-coated slides as described previously (33). Once the cells had settled, the slides were washed with cold phosphate-buffered saline (PBS) to remove any unattached cells. The attached cells were fixed with 3.7% formaldehyde and permeabilized with 0.1% Triton X-100. After blocking with 5% nonfat milk for 30 min, an anti-HA monoclonal antibody at a dilution of 1:100 in PBS was applied to the slide for 1 h. Slides were then washed with PBS containing 3% bovine serum albumin. After that, fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG was applied as a secondary antibody for visualization under a fluorescence microscope. DNA was stained with 1 μg/ml DAPI (4′,6-diamidino-2-phenylindole). Cells were imaged using a Nikon TE2000E wide-field microscope equipped with a 60×1.4 numerical aperture (NA) Plan Apo VC oil immersion objective. Images were captured using a CoolSNAP HQ2 cooled charge-coupled-device (CCD) camera and Nikon Elements Advanced Research software.

**RESULTS**

**In vitro analysis of import of TAO into mitochondria.** The putative presenence of TAO is a 24-amino-acid segment (as predicted by the Mitoprot program [19]) which lies at the N-terminal portion of the preprotein. During maturation of the protein, this preprotein is most likely cleaved between Q24 and K25 to generate the mature protein (Fig. 1A and B). To identify the region of the putative N-terminal MTS that is sufficient for the import of TAO, a series of deletion mutants were generated (Fig. 1A and B) by deleting 10 amino acids at a time from the N terminus. Figure 1C shows the pattern of migration of these mutants in a denaturing gel. A 31-kDa protein was also found in all of the *in vitro* coupled transcription-translation reactions. This species is a nonspecific product probably initiated from an internal methionine start site within TAO or in the vector itself as reported previously (26). The radiolabeled full-length and deletion mutants were then used for *in vitro* mitochondrial protein import assays (Fig. 2).

Figure 2A shows that import of the Δ10TAO mutant, which was generated by deleting the first 10 amino acids from the N terminus of the protein, was not affected, as the protein was imported and processed to a mature protein of a size similar to that of FLTAO. The time course of its import was similar to that of FLTAO (Fig. 2B). In contrast, deletion of 20 amino acids from the N terminus of TAO did not result in a smaller product (Fig. 2A), indicating that its import may have been hindered. However, given that the Δ20TAO mutant possesses only the last 4 amino acids of the predicted MTS, it seems reasonable to surmise that this amino acid sequence was too short to be recognized by the mitochondrial processing peptidase (MPP) thus not being cleaved. A similar result was obtained with the Δ30TAO mutant (data not shown). Migration of the Δ40TAO mutant in the gel was indistinguishable from that of the nonspecific protein product represented in Fig. 1C; therefore, we did not use this mutant for our *in vitro* import analysis.

Next, on the premise that membrane potential facilitates import of proteins containing N-terminal mitochondrial targeting signal into mitochondria (1, 2), we assessed the impact of disrupting membrane potential on the import of Δ10TAO mutant (Fig. 2C). To this end, mitochondria isolated from procyclic parasites were pretreated with valinomycin and CCCP before incubation with the radiolabeled precursor proteins. We found that the pre-treatment with valinomycin and CCCP reduced the import of Δ10TAO mutant proteins (Fig. 2C). As reported earlier, import of FLTAO was decreased more than 80% in the absence of mitochondrial membrane potential (26). Import of Δ10TAO was also inhibited about 50% due to disruption of mitochondrial membrane potential. This result implies that the import of the Δ10TAO mutant is relatively less dependent on mitochondrial membrane potential.

To further determine the effect of truncation of MTS on the integration of TAO into the membrane, mitochondria were subjected to alkali treatment after import of the radiolabeled precursors (Fig. 2C). The processed products generated during *in vitro* import from FLTAO and Δ10TAO were found in the alkali-resistant fraction of mitochondria, suggesting that truncation of TAO MTS by deleting 10 amino acids from the N terminus did not prevent the integration of the mature form of the protein into the mitochondrial membrane. Given that the Δ10TAO mutant was imported, properly processed, and integrated into the mitochondrial membrane in the same manner as the FLTAO protein, we conclude that the first 10 amino acids of TAO are dispensable for its import into mitochondria of the procyclic form.

![Figure 1](https://example.com/figure1.png)

**FIG 1** Generation of N-terminal deletion mutants of TAO. (A) Schematic of the full-length TAO precursor (FLTAO) and its four deletion mutants (Δ10TAO, Δ20TAO, Δ30TAO, and Δ40TAO). The predicted N-terminal MTS is shown in red. Note that the proteins are not drawn to scale. (B) The protein sequences of the N terminus of FLTAO, Δ10TAO, Δ20TAO, Δ30TAO, and Δ40TAO. Amino acid residues within the predicted MTS are in red except for the arginine (R) at position −2 from the cleavage site, which is in blue. (C) Analysis of the radiolabeled FL-, Δ10-, Δ20-, Δ30-, and Δ40TAO proteins. The FLTAO and mutant TAO proteins were synthesized in a coupled transcription-translation system in the presence of [35S]-methionine and analyzed by SDS-PAGE and autoradiography. The molecular sizes of the marker proteins are indicated. Truncated TAO proteins were generated at the anticipated sizes. A 31-kDa nonspecific protein band was also detected in all samples which could have been the result of an internal start site within the vector.
immunoprecipitated from the procyclic and bloodstream mitochondrial extracts, respectively (see Table S2 in the supplemental material). The peptide of TAO furthest upstream that we identified from both samples was 29KTPVWGHTQLN39. The tryptic peptide upstream of this sequence, 25KSDA28, was not detected in the mass spectra because the size was below the detection limit, and no further upstream peptides were detected. A similar set of peptides was also reported from previously published proteomic material. The peptide of TAO furthest upstream that we identified from both samples was 29KTPVWGHTQLN39. The tryptic peptide upstream of this sequence, 25KSDA28, was not detected in the mass spectra because the size was below the detection limit, and no further upstream peptides were detected. A similar set of peptides was also reported from previously published proteomic analysis (http://tritrypdb.org). Therefore, this finding supports the hypothesis that the TAO MTS is cleaved in both forms at the predicted site, which is after Q24.

**TAO possesses an internal targeting signal.** To investigate the import of mutant TAO proteins in intact cells, C-terminally tagged FLTAO and N-terminal deletion mutants were ectopically expressed in *T. brucei*. The proteins were expressed with a 3×-HA tag that would distinguish them from the endogenous TAO. The expression of the tagged protein was under the control of a Tet-On system. Upon induction with doxycycline, the proteins were detected in the whole-cell lysate by Western blotting using either anti-TAO or an anti-HA monoclonal antibody (Fig. 3). Subcellular fractionation analysis clearly showed that although the FLTAO, Δ10TAO, and Δ20TAO mutants were accumulated exclusively in the mitochondrial fraction, some of the expressed Δ30TAO and Δ40TAO was found in the cytosolic fraction in procyclic parasites (Fig. 3B to F). As controls, we used VDAC, a mitochondrial protein, and TbPFS, a cytosolic protein, to validate the quality of the subcellular fractionation. Together, these results showed that TAO can be imported into *T. brucei* mitochondria without its cleavable N-terminal presequence; however, truncation of more than 20 amino acid residues from the N terminus decreased import efficiency.

We also investigated the issue of what effect this truncation has on membrane integration of the protein. To address this issue, we applied the alkali extraction protocol used in Fig. 2C. In all cases, we found that the mutated protein was found in the membrane fraction after alkali extraction of isolated mitochondria (see Fig. S1 in the supplemental material), suggesting that deletion of the N terminus of TAO has no effect on integration of the protein into the mitochondrial membrane in the intact cell.

To support our subcellular fractionation data, we performed immunolocalization of the ectopically expressed proteins in intact *T. brucei* cells, using a monoclonal antibody against HA. The cells were costained with MitoTracker Red to visualize mitochondria and with DAPI to see nuclear and kinetoplast DNA. Using confocal microscopy, we could clearly visualize the colocalization of the expressed proteins with the MitoTracker-stained mitochondrion (Fig. 4). In addition, using a monoclonal antibody against TAO, we observed a similar colocalization of the endogenous protein with stained mitochondrion (Fig. 4). These results confirm that, in similarity to endogenous TAO and FLTAO, all of the N-terminal deletion mutants of TAO were localized within mitochondria at least in part despite the partial or complete absence of the N-terminal MTS. These results suggest that TAO harbors an internal targeting sequence which can drive its import into mitochondria.

**FIG 2** Effect of deletion of the first 10 and 20 amino acid residues from the N terminus of TAO on its import into procyclic mitochondria *in vitro*. (A) Radiolabeled TAO proteins (full length [FLTAO], with the first 10 amino acids truncated [Δ10TAO], and with the first 20 amino acids truncated [Δ20TAO]) were incubated with mitochondria isolated from the procyclic form of the parasite for various times. At different time points (1 to 20 min), equal amounts of the samples were harvested and mitochondria were pelleted by centrifugation. After washing, mitochondrial proteins were analyzed by SDS-PAGE and autoradiography. The precursor (p) and mature (m) proteins are indicated. The input lane represents 10% of the total radiolabeled proteins used for each reaction. (B) The intensities of the mature (m) proteins generated from FLTAO and Δ10TAO were quantitated by densitometric scanning and plotted as a percentage of the import at the highest point on the time scale for the respective proteins. The means and standard errors were calculated from three independent experiments. (C) *In vitro* import assays for FLTAO and Δ10TAO precursor protein using procyclic mitochondria with (+) or without (−) membrane potential (Δψ). As indicated, in separate experiments, mitochondria were also left untreated (−) or treated (+) with Na₂CO₃ (pH 11.5) postimport to separate soluble and integral membrane proteins. Relative intensities (RI) are presented as percentages of the imported protein in the untreated control as obtained by densitometric scanning.
The internal targeting signal of TAO is recognized in mitochondria of bloodstream parasites. In order to investigate if the internal MTS of TAO is functional in the bloodstream form, bloodstream cells were transfected with constructs expressing FLTAO or the Δ/H9004 40TAO mutant. In bloodstream parasites, both FLTAO and the Δ/H9004 40TAO mutant were expressed after induction with doxycycline and were detected in whole-cell extracts by the anti-HA monoclonal antibody (Fig. 5A). Subcellular fractionation experiments showed that the expressed protein was accumulated in the mitochondrial fraction in a manner similar to that seen with endogenous TAO. VDAC and TbPP5 were used as the mitochondrial and cytosolic marker proteins, respectively. In contrast to the FLTAO protein results, a small fraction of Δ/H9004 40TAO was detected in the cytosolic fraction, indicating that the mutant protein is possibly imported less efficiently than the full-length protein, leading to some accumulation in the cytosol. Anti-TAO antibody detected endogenously expressed TAO exclusively in the mitochondrial fractions. However, this antibody could not detect the ectopically expressed FLTAO and the Δ/H9004 40TAO mutant due to a lower level of expression of these proteins in the bloodstream form. Alkali extraction of mitochondrial proteins revealed that both FLTAO and Δ/H9004 40TAO are in the alkali-resistant fractions, indicating that, as seen with FLTAO, the Δ/H9004 40TAO mutant is also integrated into the mitochondrial membrane (see Fig. S1 in the supplemental material). Immunostaining with a monoclonal HA antibody followed by an FITC-conjugated secondary antibody revealed an overlap of the ectopically expressed proteins and MitoTracker-stained mitochondrion, which further validated the localization of both FLTAO and Δ/H9004 40TAO in mitochondria (Fig. 5B). Overall, these results show that, as seen with the procyclic form, TAO is imported into mitochondria in the bloodstream parasite without the N-terminal MTS.

**N-terminal and internal targeting signals of TAO can function independently.** To determine if the N-terminal MTS and internal MTS of TAO function independently, we fused DHFR to the first 30 amino acids of TAO, as well as to the Δ/H9004 30TAO mutant; these fusion constructs are designated (1-30)TAO-DHFR and Δ/H9004 30TAO-DHFR, respectively (Fig. 6A). All three fusion proteins were tagged at their C-terminal ends with 3X-HA tag. Anti-HA antibody readily detected all three expressed proteins in the total cell extract at the expected molecular sizes of approximately 60 kDa, 59 kDa, and 25 kDa for TAO-DHFR, Δ/H9004 30TAO-DHFR, and (1-30)TAO-DHFR, respectively (Fig. 6B). Subcellular fractionation analysis showed...
that TAO-DHFR and Δ30TAO-DHFR accumulated in the mitochondrial fraction. Although (1-30)TAO-DHFR was also targeted to mitochondria, a larger portion of this chimeric protein was detected in the cytosolic fraction (Fig. 6B). On the other hand, while we expressed DHFR alone with a 3×-HA tag, we found that the expressed protein accumulated in the cytosolic fraction in T. brucei as expected (Fig. 6B). We interpret this to mean that the internal mitochondrial targeting signal of TAO is more efficient than its N-terminal MTS counterpart at targeting a heterologous protein to mitochondria.

Alkali extraction of mitochondrial proteins showed that the Δ30TAO-DHFR fusion protein was assembled in the mitochondrial membrane, whereas (1-30)TAO-DHFR was found as a soluble mitochondrial protein (see Fig. S1 in the supplemental material). This is not surprising given that (1-30)TAO-DHFR lacks the membrane-spanning region. Immunostaining with anti-HA antibody followed by an FITC-conjugated secondary antibody revealed expression of the fusion proteins. The overlapping of confocal images for FITC- and MitoTracker-stained T. brucei indicated that the fusion proteins were localized in mitochondria (Fig. 7). In support of our subcellular fractionation analysis, some cytosolic localization of (1-30)TAO-DHFR was also observed. All together, these results showed that TAO possess a validated N-terminal MTS within the first 30 amino acid residues, as well as one or more internal targeting signals within Δ30TAO.

The internal targeting sequence of TAO is mapped within amino acid residues 115 to 146 of the protein. In silico analysis of the TAO fragments using the Mitoprot program identified two regions within the mature part of TAO possessing the characteristics of the presequence (Fig. 8A). One region is within amino acid residues 100 to 146, and the other is located within residues 170 to 210 (see Table S3 in the supplemental material). Because the probability score for mitochondrial targeting was higher for the former region than for the latter region, we constructed a fusion protein consisting of DHFR linked at the N terminus to TAO (TAO-DHFR), the first 30 amino acids of TAO with DHFR [(1-30)TAO-DHFR], and the N-terminal 30-amino-acid-deletion mutant of TAO with DHFR (Δ30TAO-DHFR). Each of these chimeric proteins possesses a C-terminal 3×-HA tag (shown in blue). The presequences in TAO-DHFR and (1-30) TAO-DHFR are shown in red. After induction of expression of these fusion proteins for 48 h using doxycycline, total cell extracts (T), cytosol (C), and mitochondria (M) were analyzed by SDS-PAGE and Western blotting using antibodies against HA, TAO, VDAC, and TbPP5. Protein from each fraction was loaded in each lane in equal amounts. (B) T. brucei bloodstream cells containing FLTAO and the Δ40TAO deletion construct and grown in the presence of doxycycline for 48 h were stained with MitoTracker Red followed by immunostaining with anti-HA monoclonal antibody and an FITC-conjugated secondary antibody. DAPI was used to visualize nuclear and kinetoplast DNA. Images were taken by confocal microscopy. FITC (green), MitoTracker (red), and DAPI (blue) images from the same cells were merged to show colocalization.

FIG 6 Expression, subcellular localization, and alkali extraction of TAO-DHFR proteins in T. brucei procyclic form. (A) Schematics of TAO-DHFR fusion proteins (N-terminal MTS shown in red; DHFR represented by shaded box), including full-length TAO fused with DHFR (TAO-DHFR), the first 30 amino acids of TAO with DHFR [(1-30)TAO-DHFR], and the N-terminal 30-amino-acid-deletion mutant of TAO with DHFR (Δ30TAO-DHFR). Each of these chimeric proteins possesses a C-terminal 3×-HA tag (shown in blue). The presequences in TAO-DHFR and (1-30) TAO-DHFR are shown in red. (B) After induction of expression of these fusion proteins for 48 h using doxycycline, total cell extracts (T), cytosol (C), and mitochondria (M) were analyzed by SDS-PAGE and immunoblot analysis using antibodies against HA, TAO, VDAC, and TbPP5. The chimeric TAO proteins (TAO-DHFR and Δ30TAO-DHFR) were recognized by anti-TAO as well as by anti-HA antibodies, and (1-30)TAO-DHFR was detected by anti-HA antibody.
In this report, we show that TAO is imported into the mitochondrion of *T. brucei* in the absence of its canonical N-terminal MTS, suggesting that an additional targeting signal(s) is present within the mature TAO protein. We identified an internal signal sequence of TAO that is located within amino acid residues 115 to 146. This internal targeting signal of TAO can function independently and could drive the import of a heterologous nonmitochondrial protein to the organelle. Both the N-terminal MTS and the internal signals are functional for import of TAO into the *T.*

**FIG 7** Immunolocalization of TAO-DHFR proteins in *T. brucei* procyclic form. *T. brucei* procyclic cells containing TAO-DHFR, (1-30)TAO-DHFR, or Δ30TAO-DHFR fusion constructs were grown in the presence of doxycycline for 48 h, and cells were stained with MitoTracker Red followed by immunostaining with anti-HA monoclonal antibody and FITC-conjugated secondary antibody. DAPI was used to visualize nuclear and kinetoplast DNA. Images were taken by confocal microscopy. FITC (green), MitoTracker (red), and DAPI (blue) images from the same cells were merged to show colocalization.

**FIG 8** Subcellular localization of (115-146)TAO-DHFR in procyclic cells. (A and B) Schematics of TAO proteins with two putative transmembrane domains (TM1 and TM2) (A) and the (115-146)TAO-DHFR construct (B). The approximate size of the fusion protein is 30 kDa. (C) Parasites were fractionated after 48 h of induction, and total (T), cytosolic (C), and mitochondrial (M) fractions were analyzed by SDS-PAGE and immunoblotting using antibodies against HA, TAO, VDAC, and TbPP5. (D) *T. brucei* procyclic cells containing (115-146)TAO-DHFR grown in the presence of doxycycline for 48 h were stained with MitoTracker Red followed by immunostaining with anti-HA monoclonal antibody and FITC-conjugated secondary antibody. DAPI was used to visualize nuclear and kinetoplast DNA. Images were taken by confocal microscopy. FITC (green), MitoTracker (red), and DAPI (blue) images from the same cells were merged to show colocalization.
brucei mitochondrion. The chemical nature of the TAO internal signal is very similar to that of the N-terminal MTS and contains an appropriate mixture of hydrophobic and charged residues. Although not experimentally proven, a similar region is also found within the second transmembrane domain of TAO, suggesting that TAO possesses multiple internal targeting signals along with its N-terminal MTS.

TAO is a developmentally regulated protein, and its expression is upregulated in the bloodstream mitochondrion at the same time that many other mitochondrial activities are suppressed (16). However, the effects of N-terminal truncation on subcellular localization of TAO were very comparable in the procyclic form and bloodstream form, suggesting that the internal signal(s) of TAO is equally operative in both forms of T. brucei. Therefore, TAO is imported by similar mechanisms in the two developmental forms.

It has been reported recently that some hydrogenosomal proteins in Trichomonas vaginalis contain internal targeting signals in addition to a validated N-terminal MTS (34). Hydrogenosomes are double membrane-bound organelles related to mitochondria (35). As seen with a number of trypanosome mitochondrial proteins, many of the hydrogenosome proteins possess a relatively short cleavable N-terminal MTS (36). In addition, recent evidence indicated that these signals are often not required for the import of these proteins into hydrogenosomes (34). Instead, internal targeting signals located inside the coding regions are capable of importing these proteins. Although this internal signal has not yet been characterized, it appears that import of proteins into mitochondria and hydrogenosomes often depends more on internal than on N-terminal MTS.

In fungi, there are a few mitochondrial inner membrane proteins which possess similar presequence-like internal targeting signals besides its N-terminal MTS, such as cyt c1 (37, 38). However, unlike TAO, this internal targeting signal of cyt c1 is located downstream of its single transmembrane domain. Although the import pathway is controversial, the bipartite N-terminal MTS and the internal MTS of cyt c1 are required together for proper intramitochondrial localization of cyt c1. Another fungal protein, Bcs1, which is involved in the assembly of the bc1 complex in the mitochondrial inner membrane, also possesses a presequence-like internal targeting signal within the N-terminal half of the protein; however, this protein does not have any cleavable N-MTS (39, 40). It is speculated that the entire N-terminal domain of Bcs1 forms a loop structure and that the internal targeting signal is thus exposed and recognized by Tom and Tim proteins. This loop structure also helps the integration of this protein into the mitochondrial inner membrane in proper orientation.

Whether TAO can be imported via a similar mechanism remains unknown. In fact, because of the paucity of information on trypanosomatid mitochondrial protein import machinery, it is difficult at this time to assess the mechanistic details of the import pathway of TAO in T. brucei. It can be speculated that ATOM (archaic translocase of the outer mitochondrial membrane), a functional homolog of Tom40 in the T. brucei mitochondrial outer membrane (5), mediates translocation of TAO through mitochondrial outer membrane. ATOM36 (41), a novel protein of the T. brucei mitochondrial outer membrane, was shown to be responsible for import of presequence-containing proteins. Therefore, this protein may also be involved in recognition and translocation of the N-terminal MTS as well as the presequence-like internal targeting signal(s) of TAO. However, we cannot exclude the possibility that different receptor proteins are responsible for recognition of two different signals in TAO. We have shown previously that the TbTim17 and the newly identified Tb-Tim17-associated proteins TbTim62, TbTim54, and TbTim50 are critical for import of TAO into mitochondria (4, 42), which suggests that TAO is imported via a protein complex containing these Tbtim proteins. Therefore, it is clear that the uniquely orchestrated import process of TAO depends on several novel components of the protein import machinery in T. brucei. The complete picture of TAO import will be revealed only after further investigation.

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