Functional Complementation Analyses Reveal that the Single PRAT Family Protein of Trypanosoma brucei Is a Divergent Homolog of Tim17 in Saccharomyces cerevisiae

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Trypanosoma brucei, a parasitic protozoan that causes African trypanosomiasis, possesses a single member of the presequence and amino acid transporter (PRAT) protein family, which is referred to as TbTim17. In contrast, three homologous proteins, ScTim23, ScTim17, and ScTim22, are found in Saccharomyces cerevisiae and higher eukaryotes. Here, we show that TbTim17 cannot rescue Tim17, Tim23, or Tim22 mutants of S. cerevisiae. We expressed S. cerevisiae Tim23, Tim17, and Tim22 in T. brucei. These heterologous proteins were properly imported into mitochondria in the parasite. Further analysis revealed that although ScTim23 and ScTim17 were integrated into the mitochondrial inner membrane and assembled into a protein complex similar in size to TbTim17, only ScTim17 was stably associated with TbTim17. In contrast, ScTim22 existed as a protease-sensitive soluble protein in the T. brucei mitochondrion. In addition, the growth defect caused by TbTim17 knockdown in T. brucei was partially restored by the expression of ScTim17 but not by the expression of either ScTim23 or ScTim22, whereas the expression of TbTim17 fully complemented the growth defect caused by TbTim17 knockdown, as anticipated. Similar to the findings for cell growth, the defect in the import of mitochondrial proteins due to depletion of TbTim17 was in part restored by the expression of ScTim17 but was not complemented by the expression of either ScTim23 or ScTim22. Together, these results suggest that TbTim17 is divergent compared to ScTim23 but that its function is closer to that of ScTim17. In addition, ScTim22 could not be sorted properly in the T. brucei mitochondrion and thus failed to complement the function of TbTim17.

A majority of proteins in the mitochondria are encoded by nuclear DNA. These proteins are imported by the translocase of the mitochondrial outer membrane (TOM) and the translocase of the mitochondrial inner membrane (TIM) (1, 2). The TOMs and TIMs are multiprotein complexes whose structure and function have been extensively characterized in fungi and recently in humans and plants. The TOM complex serves as the entry gate for virtually all mitochondrial proteins (3). There are two TIM complexes, TIM23 and TIM22, in the majority of eukaryotes analyzed so far. Unlike TOM, the TIM complexes have substrate specificities. The TIM23 complex imports proteins that contain an N-terminal targeting signal (MTS) into the mitochondrial matrix and, if they contain an additional sorting signal, into the inner membrane (4, 5). Tim23 and Tim17, together with the receptor Tim50, form the core of the TIM23 complex. This core complex seems to be sufficient for transport of proteins into the mitochondrial inner membrane by a stop-transfer pathway. For translocation of proteins to the mitochondrial matrix, the ATP-dependent action of the import motor of the TIM23 complex is additionally required (4–6). The TIM22 complex, on the other hand, is involved in the translocation and insertion of a special class of mitochondrial inner membrane proteins. These proteins have multiple internal targeting signals, such as mitochondrial metabolite carrier proteins (MCPs) (7, 8). The membrane-embedded part of TIM22 consists of Tim22, Tim54, and Tim18. Tim12 is an additional component of this complex and aids with the docking of MCPs carried by the small Tim proteins (Tim9 and Tim10) in the intermembrane space (IMS) (9).

Tim17, Tim23, and Tim22 all belong to the presequence and amino acid transporter (PRAT) protein family (10, 11). These proteins have 4 transmembrane domains located at the center and possess a PRAT signature motif [(G/A)X_2(F/Y)X_5(R/K/D)X_3D(G/A/S)GX_3(G)]. In spite of the similarity of their secondary structure, these proteins perform distinct functions. Saccharomyces cerevisiae Tim23 (ScTim23) and ScTim22 form a protein import channel in the TIM23 and TIM22 complexes, respectively (12, 13). The function of ScTim17 is less clear; it seems to act as the structural component that is involved in gating the Tim23 channel (14). Homologs of these Tim proteins are also present in human and plants (15–18).

Trypanosoma brucei belongs to a group of ancient and divergent eukaryotes. It possesses a single mitochondrion with many unique and essential activities (19–21). Similar to other eukaryotes, hundreds of nuclear DNA-encoded proteins are imported into the T. brucei mitochondrion. However, T. brucei possesses relict import machinery for mitochondrial proteins (22). There is no canonical TOM complex in this parasite. Instead, an archaic protein termed ATOM imports proteins across the outer mitochondrial membrane (23, 24). T. brucei possesses a single
PRAT family protein named \textit{T. brucei} Tim17 (TbTim17) (25, 26) and also has a homolog of Tim50 (27). Three small Tim proteins have been identified in \textit{T. brucei}; however, their functions have not been characterized as yet. TbTim17 is localized in the mitochondrial inner membrane. It is essential for the import of proteins into the mitochondrion and for cell survival (25, 26). We recently showed that TbTim17 is present in a larger protein complex of 1,100 kDa (28). TbTim17 is also found to be associated with several trypanosome-specific proteins (TbTim62, TbTim54) that are involved in mitochondrial protein import (28). In addition, recent evidence indicates that TbTim17 is involved in the import of tRNAs into the \textit{T. brucei} mitochondrion (29). Although \textit{T. brucei} possesses a large set of mitochondrial metabolite carrier proteins, the homologs of any of the components of the mitochondrial carrier translocase TIM22 have not been detected in trypanosome genome databases. However, it is speculated that TbTim17 along with small TbTim proteins perform this function (22).

TbTim17 possesses 4 predicted transmembrane domains and has an ~20 to 30% primary sequence similarity with fungal Tim23, Tim17, and Tim22 (25). However, the functional equivalent of TbTim17 among these three proteins has not been determined. Here, we performed a functional complementation analysis of TbTim17 with Saccharomyces cerevisiae Tim17, Tim23, and Tim22 and vice versa. We show that the function of TbTim17 is partially complemented by the expression of ScTim17 but not by the expression of ScTim23 or ScTim22. All three proteins were targeted to the mitochondrion in \textit{T. brucei}. ScTim23 and ScTim17, but not ScTim22, were integrated into the mitochondrial inner membrane. ScTim23 and ScTim17 also assembled into a protein complex similar in size to one of the TbTim17 protein complexes; however, only ScTim17 was stably associated with TbTim17 and participated in mitochondrial protein import. Therefore, these results indicate that TbTim17 is functionally closer to ScTim17.

**MATERIALS AND METHODS**

**\textit{T. brucei} strains, media, and cell growth.** The procyclic form of cells of the \textit{T. brucei} 427 double-resistant 29-13 cell line expressing a tetracycline repressor gene and a T7 RNA polymerase was grown in Schneider’s Drosophila medium supplemented with 10% fetal bovine serum and antibiotics (50 \mu g/ml hygromycin and 15 \mu g/ml G418) (30). To measure cell growth, cells were seeded at a density of 2 \times 10^6 cells/ml in fresh medium containing the appropriate antibiotics. Cells were harvested at different time points (0 to 9 days), and cell numbers were counted using a Neubauer hemocytometer. The log of the cumulative cell numbers versus the time (days) of incubation was plotted.

**Generation of plasmid constructs and transfection.** The open reading frames (ORFs) for ScTim17, ScTim23, and ScTim22 were PCR amplified using the corresponding cDNA clones as the templates and sequence-specific primers. The forward and reverse primers were designed to add the nucleotide sequence-encoded 23\’-myc epitope (EQKLLISEDEL) was added to the 5\’ end of the reverse primers (see Table S1 in the supplemental material) for inductive expression of ScTim17, ScTim23, and ScTim22 with a 2\times myc tag at the C-terminal end. For generation of TbTim17 double-stranded RNA targeted to the 3\’ untranslated region (UTR) of the TbTim17 transcript, the 3\’ UTR of TbTim17 was PCR amplified from \textit{T. brucei} genomic DNA using the forward and reverse primers containing the restriction sites BamHI and HindIII, respectively, at the 5\’ ends (see Table S1 in the supplemental material). The 476-bp amplicon was subcloned into the multiple-cloning site of the tetracycline-inducible (Tt) T7 double-headed promoter plasmid vector p2T7\(^{51,177}\) (31) between the BamHI and HindIII restriction enzyme sites. After transfection, cells were selected by phleomycin (2.5 \mu g/ml). A wild-type TbTim17 construct was also generated by subcloning the open reading frame of TbTim17 into the pHID-1344 vector as described above for the \textit{S. cerevisiae} yeast proteins. Plasmid DNAs were linearized by NotI and transfected into cells of the TbTim17 3\’ UTR RNAi cell line, and the transfected cells were selected by puromycin (1 \mu g/ml).

**Subcellular fractionation.** Fractionation of \textit{T. brucei} procyclic cells was performed as described previously (25, 27). Briefly, 2 \times 10^7 cells were resuspended in 500 \mu l of SMEP buffer (250 mM sucrose, 20 mM MOPS [morpholinepropanesulfonic acid]-KOH, pH 7.4, 2 mM EDTA) containing 0.03% digitonin and incubated on ice for 5 min. The cell suspension was then centrifuged for 5 min at 6,800 \times g and 4°C. The resultant pellet was considered a crude mitochondrial fraction, and the supernatant contained soluble cytosolic proteins.

**Isolation and postisolation treatments of mitochondria.** Mitochondria were also isolated from the parasite after lysis via nitrogen cavitation in isotonic buffer as described previously (25, 27). The isolated mitochondria were stored at a protein concentration of 10 mg/ml in SME buffer (250 mM sucrose, 20 mM MOPS-KOH, pH 7.4, 2 mM EDTA) containing 50% glycerol at ~70°C. For limited protease K (PK) digestion, mitochondria in SME buffer (1 mg/ml) were treated with various concentrations of PK (0 to 150 \mu g/ml) for 30 min on ice. After incubation, the PK was inhibited by PMSF (2 mM) and the mitochondria were resolubilized by centrifugation at 10,000 \times g and 4°C for 10 min. For alkali extraction, mitochondria (100 \mu g) isolated from \textit{T. brucei} were treated with 100 mM Na\(_2\)CO\(_3\) (100 \mu M) at pH 11.5 for 30 min on ice (25, 27). The supernatant and pellet fractions were collected after centrifugation and analyzed by SDS-PAGE and immunoblotting.

**Mitochondrial protein import assay in vivo.** Cells with TbTim17 RNAi containing a genome-integrated copy of the mitochondrial RNA-binding protein MRP2 with a 2\times myc tag (MRP2-2\times myc) at the C terminus were previously developed in our laboratory to study the effect of TbTim17 RNAi on \textit{in vivo} targeting of MRP2 (28). Cells of this cell line (MRP2-2\times myc/Tim17 RNAi) were further transfected with either the pHID1344-ScTim17, pHID1344-ScTim23, or pHID1344-ScTim22 construct, and cells were selected by puromycin as described above. These stably transfected cells were cultured in the presence of hygromycin (50 \mu g/ml), G418 (15 \mu g/ml), phleomycin (2.5 \mu g/ml), blasticidin (10 \mu g/ml), and puromycin (1 \mu g/ml) to maintain the respective constructs integrated into the parasite genome. Cells were then induced with doxycycline for expression of MRP2-2\times myc as well as TbTim17 double-stranded RNA. \textit{T. brucei} cells transfected only with pLew100-Bsd-MRP2-2\times myc were used as controls. At different time points after induction with doxycycline, cells were lysed and the mitochondrial and cytosolic fractions were separated. Equal amounts of proteins from the mitochondrial fractions were analyzed by SDS-PAGE and immunoblotting to assess the level of MRP2-2\times myc in the mitochondria.

**Yeast complementation.** \textit{T. brucei} Tim17 was cloned in yeast expression vector pYT-U, which enabled the expression of cloned genes under the control of the constitutive alcohol dehydrogenase promoter (32). To test complementation in yeast, plasmids were transformed into the yeast strains GAL-Tim23, GAL-Tim17, and GAL-Tim22 using a lithium acetate method (33, 34). Empty plasmids and plasmids containing ScTim23, ScTim17, and ScTim22 were transformed as controls. The yeast strains GAL-Tim23, GAL-Tim17, and GAL-Tim22, expressing ScTim17,
ScTim23, and ScTim22, respectively, under the control of the GAL promoter, have been previously described (35). Since these Tim proteins are essential, all of these strains require the presence of galactose (Gal) in the growth medium. The ability of T. brucei Tim17 to substitute for ScTim23, ScTim17, or ScTim22 was therefore assessed using media lacking or containing 0.5% (wt/vol) Gal. Two independent transformants were analyzed for each transformation.

**SDS-PAGE and Western blot analysis.** Proteins from whole cells or isolated mitochondria were separated on an SDS-polyacrylamide gel and immunoblotted with polyclonal antibodies for the myc epitope (Abcam), TbTim17 (25), T. brucei voltage-dependent anion channel protein (VDAC) (36), T. brucei serine/threonine protein phosphatase 5 (TbPP5) (37), T. brucei mitochondrial RNA-binding protein RBP16 (38), and T. brucei mitochondrial heat shock protein 70 (mHsp70) (39) or monoclonal antibodies for trypanosome alternative oxidase (TAO) (40) and T. brucei/H9252-tubulin (41). Blots were developed with appropriate secondary antibodies and an enhanced chemiluminescence (ECL) kit (Pierce).

**Blue native (BN) PAGE analysis.** Mitochondrial proteins (100 μg) were solubilized in 50 μl of an ice-cold native buffer (50 mM NaCl, 7.2, 50 mM NaCl, 10% [wt/vol] glycerol, 1 mM PMSF, 1 mM/ml leupeptin, 1% digitonin). The solubilized mitochondrial proteins were clarified by centrifugation at 100,000 x g for 30 min at 4°C. The supernatants were electrophoresed on a precast (4 to 16%) bis-Tris polyacrylamide gel (Invitrogen) according to the manufacturer’s protocol. Protein complexes were detected by immunoblot analysis. Molecular size marker proteins apoferritin dimer (886 kDa), apoferritin monomer (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), and bovine serum albumin (66 kDa) were electrophoresed on the same gel and visualized by Coomassie staining.

**Coimmunoprecipitation (co-IP).** Mitochondrial proteins (200 μg) isolated from T. brucei wild type and those expressing ScTim23-2 myc, ScTim17-2 myc, and TbTim17-2 myc were solubilized with native buffer (200 μl). Solubilized proteins were then subjected to immunoprecipitation with anti-myc-conjugated Sepharose beads at 4°C overnight. The beads were washed sequentially with the same buffer, and specifically bound proteins were eluted with 2 Laemmli buffer and analyzed by SDS-PAGE and immunoblotting.

**RESULTS**

Comparison of primary and secondary structures of TbTim17 with its homologs in yeast. TbTim17 possesses a PRAT motif within amino acid residues 83 to 113 (Fig. 1A). Multiple-sequence alignment of TbTim17 with ScTim23, ScTim17, and ScTim22 using the Clustal W program (42) revealed that the PRAT motif is located within the C-terminal half of each of these proteins and is in a largely conserved region (Fig. 1A). In contrast, the N-terminal regions are more divergent. A dendrogram analysis showed that TbTim17 is closer to ScTim22 and ScTim17 than to ScTim23 (see Fig. S1A in the supplemental material). The primary sequence of TbTim17 showed 21.8, 29.7, and 31.2% similarity and 7.1, 18, and 16.8% identity with the primary sequences of ScTim23, ScTim17, and ScTim22, respectively (see Fig. S1B in the supplemental material). The secondary structure predicted by the TMPred server (43) indicated that TbTim17 possesses 4 transmembrane domains (TMs; TM1 to TM4). Blots were developed with appropriate secondary antibodies and an enhanced chemiluminescence (ECL) kit (Pierce).
minal hydrophilic region of TbTim17 is relatively longer than that seen in ScTim23 but shorter than that in ScTim17. Therefore, by bioinformatics analysis alone, it is difficult to assess which of these three fungal Tim proteins is functionally closer to TbTim17. Conditional mutants of ScTim17, ScTim23, and ScTim22 cannot be rescued by TbTim17. Next, we evaluated if TbTim17 could complement the growth of yeast strains depleted of either Tim23, Tim17, or Tim22. Since these ScTim proteins are essential, the GAL-Tim17, GAL-Tim23, and GAL-Tim22 yeast strains were used for the complementation analysis. In these cells the endogenous copy of the respective gene was placed under the control of the GAL promoter so that yeast cells could survive only in the presence of galactose. Transformation with an expression vector containing TbTim17 or the empty vector did not allow these cells to grow in a galactose-free medium (Fig. 2A). However, transformation with the same vector containing the respective genes for yeast Tim proteins clearly rescued the growth in the galactose-free medium. These results indicate that TbTim17 is unable to functionally complement either ScTim23, ScTim17, or ScTim22. To analyze the expression of TbTim17 in yeast, the yeast strains depicted in Fig. 2 were grown in the presence of galactose, and the mitochondrial and cytosolic fractions were isolated as described previously (44). Analysis of proteins from these fractions revealed that TbTim17 was expressed at comparable levels and was properly targeted to the mitochondria in all yeast strains (Fig. 2B). The yeast mitochondrial escape 1 (Yme1) protein was used as a marker for the mitochondrial fractions. These results indicate that TbTim17 can be stably expressed in yeast mitochondria; however, it likely cannot properly associate with other components of the TIM23 and TIM22 complexes.

Ectopically expressed ScTim17, ScTim23, and ScTim22 are targeted to T. brucei mitochondria. To assess if S. cerevisiae Tim17, Tim23, and Tim22 can be expressed and properly targeted to mitochondria in T. brucei, we transfected the procyclic form of T. brucei with the C-terminally 2× myc-tagged constructs of the ScTim proteins. Analysis of total cellular proteins by Western blot analysis using an anti-myc antibody showed that the S. cerevisiae proteins were expressed with the anticipated sizes upon induction.
with doxycycline (Fig. 3A). We did not observe any changes in growth pattern due to expression of these heterologous proteins in *T. brucei* (see Fig. S2A in the supplemental material). In addition, the expression levels of these ScTim proteins were consistent for at least 6 days postinduction (see Fig. S2B in the supplemental material). To determine the subcellular location of the heterologous proteins in *T. brucei*, we separated the cytosolic and mitochondrial fractions from the cell lysates. Analysis of the fractions revealed that ScTim23, ScTim17, and ScTim22 were enriched in the mitochondrial fractions from the cell lysates. Analysis of the fractions revealed that ScTim23, ScTim17, and ScTim22 were enriched in the mitochondrial fractions of *T. brucei* (Fig. 3B). A mitochondrial protein, VDAC, and a cytosolic protein, *T. brucei* PPS (TbPP5), were used as markers for the corresponding fractions.

ScTim17 and ScTim23 are integrated into the mitochondrial inner membrane in *T. brucei*. Limited protease digestion of the isolated mitochondria was performed to further analyze the subcellular location of the heterologous proteins in *T. brucei*. Analysis of mitochondrial proteins after protease digestion revealed that ScTim23 and ScTim17 were protected even after treatment with 150 μg/ml of PK (Fig. 4A and B). However, both ScTim23 and ScTim17 were completely digested when mitochondrial membranes were solubilized by Triton X-100, showing that these proteins are not per se resistant to PK. A *T. brucei* mitochondrial inner membrane protein (TbTim17), an outer membrane protein (VDAC), and a matrix protein (mHsp70) were used as controls. TbTim17 and mHsp70 were protected from PK digestion in all mitochondrial samples, as expected. Although VDAC is an outer membrane protein, this β-barrel protein is mostly embedded in the membrane and is thus protected upon PK treatment. Furthermore, tubulin is not a mitochondrial protein. It is present in the *T. brucei* mitochondrial preparation as a peripherally associated protein. Due to the unavailability of a suitable marker protein that is exposed to the cytosolic face of the mitochondrial outer membrane in *T. brucei*, we used an antitubulin antibody to test the level of this protein in our samples before and after PK treatment. We found that the level of tubulin was reduced after treatment with PK even at 50 μg/ml, indicating that protease treatment digested only the peripherally associated outer membrane proteins. Taking the findings altogether, we concluded that ScTim23 and ScTim17 are located in the mitochondrial inner membrane in *T. brucei*. In contrast to ScTim23 and ScTim17, ScTim22 was sensitive to PK even at a very low concentration (such as 25 μg/ml) (Fig. 4C), whereas VDAC, TbTim17, and mHsp70 were protected from PK treatment. Therefore, these results suggest that although ScTim22 is targeted to *T. brucei* mitochondria, it is not localized in the mitochondrial inner membrane.

Next, we investigated if ScTim23, ScTim22, and ScTim17 were integrated into mitochondrial membranes in *T. brucei*. Mitochondria isolated from *T. brucei* expressing the 2× myc-tagged copies of ScTim23, ScTim22, and ScTim17 were extracted with Na2CO3, and the proteins in the soluble and pelleted fractions were analyzed. ScTim23 and ScTim17 were mostly found in the pellet fractions, as was the endogenous mitochondrial membrane protein VDAC (Fig. 4D). RBP16, a soluble matrix protein in *T. brucei* mitochondria, was found in the soluble fraction, as expected. Interestingly, ScTim22 was mostly fractionated as a soluble protein, suggesting that ScTim22 failed to be integrated into the mitochondrial membrane of *T. brucei*.

ScTim23 and ScTim17 are assembled into protein complexes similar in size to TbTim17 in *T. brucei*. To investigate if ScTim23 and ScTim17 were assembled in the TbTim17 protein complex in

![FIG 4](https://example.com/figure4.png) Intramitochondrial location of ScTim23-2× myc, ScTim17-2× myc, and ScTim22-2× myc in *T. brucei*. (A to C) Limited protease digestion of mitochondria (100 μg) isolated from *T. brucei* expressing ScTim23, ScTim17, or ScTim22. Mitochondria were treated with various concentrations (0 to 150 μg/ml) of proteinase K (Prot. K), as described in Materials and Methods. Proteins were analyzed by immunoblotting using anti-myc antibodies. Antibodies for TbTim17, VDAC, mHsp70, and tubulin were used as controls. As indicated, some samples were also treated with Triton X-100 (1%) along with proteinase K. (D) Alkali extraction of mitochondria isolated from *T. brucei* expressing ScTim23-2× myc, ScTim17-2× myc, or ScTim22-2× myc. Proteins from equal volumes of the supernatant (lanes S) and pelleted (lanes P) fractions were analyzed by immunoblotting using anti-myc, anti-RBP16, and anti-VDAC antibodies.
*T. brucei*, BN-PAGE analysis was performed. Mitochondria isolated from *T. brucei* expressing ScTim23-2×myc, ScTim17-2×myc, or ScTim22-2×myc were solubilized with 1% digitonin. Mitochondria from the wild-type and the TbTim17-knockdown (KD) *T. brucei* strains were used in parallel as a positive control and a negative control, respectively. After analysis of the mitochondrial extract on a BN-polyacrylamide gel, TbTim17 protein complexes were detected by immunoblot analysis using anti-TbTim17 antibody. A duplicate blot was probed with anti-myc antibody for detection of potential ScTim23 and ScTim17 protein complexes. Anti-TbTim17 antibody detected two protein complexes from mitochondrial extracts obtained from the *T. brucei* wild type within a molecular size range of from 300 to 1,100 kDa (Fig. 5A). The lower protein band had a relatively broader molecular size (300 to 400 kDa) than the larger complex (~1,100 kDa). Similar complexes were also observed in the mitochondrial extracts from those cells expressing ScTim23-2×myc, ScTim17-2×myc, and ScTim22-2×myc. Furthermore, both of these protein complexes were significantly reduced in TbTim17-KD mitochondria, indicating the presence of TbTim17 in these complexes. We reported previously that TbTim17 is present in a megadalton-size complex (28). Using a higher concentration of the anti-TbTim17 antibody and a standardized precast gel, we have now identified 2 distinct complexes for TbTim17. The anti-myc antibody specifically detected an ~300-kDa protein complex in the mitochondrial extract obtained from *T. brucei* expressing ScTim23-2×myc and ScTim17-2×myc but not from the wild-type mitochondria or mitochondria from which TbTim17 was depleted (Fig. 5A), indicating that these heterologous proteins are assembled in this complex, which is similar in size to that of one of the complexes formed by TbTim17. Interestingly, this complex was not detected by the anti-myc antibody in the mitochondrial extract obtained from *T. brucei* expressing ScTim22-2×myc, which supports our previous observation that although ScTim22 is targeted to mitochondria, it is not integrated into the mitochondrial membrane and it is not assembled into a membrane complex similar to ScTim23 and ScTim17. The anti-myc antibody did show some cross-reactivity with other protein complexes (~600 kDa and larger). Since these complexes were also detected at similar intensities in wild-type mitochondria using the anti-myc antibody, we did not consider them to be specific complexes formed by ScTim proteins. Although the intensity of these nonspecifically interacting protein bands was much weaker in TbTim17-KD mitochondrial samples, this could be due to a decrease in the nonspecifically interacting proteins in these mitochondria. In contrast, VDAC protein complexes were detected in all samples showing equal loading.

To further analyze the possible association of ScTim23, ScTim17, and ScTim22 with TbTim17 in *T. brucei*, co-IP experiments were performed. Anti-myc antibody pulled down ScTim23, ScTim17, and ScTim22 from the respective mitochondrial extracts (Fig. 5B). This antibody did not detect any protein in the immunoprecipitate from wild-type mitochondrial extracts. Probing of this blot with anti-TbTim17 antibody showed that TbTim17 was coimmunoprecipitated with ScTim17 but not with ScTim23 from the respective mitochondrial extract. We found that the TbTim17 level in the input lane for the wild-type control was comparable to that in other samples except for those containing ScTim23-myc mitochondria. We observed that the TbTim17 level was upregulated in these cells. Although the reason for this upregulation is not clear at present, we speculate that expression of ScTim23 is inhibitory for TbTim17 function and, to compensate for this effect, the TbTim17 levels were increased. In spite of the presence of a large amount of TbTim17 in the mitochondrial extract, this protein was not found in the immunoprecipitate, showing that ScTim23 is not stably associated with TbTim17. On the other hand, a large proportion of TbTim17 was found to be associated with ScTim17, indicating that these two proteins interact in a stable manner in *T. brucei*. A trace amount of TbTim17 was found in the immunoprecipitate from the wild-type and ScTim22 mitochondrial extracts; however, this could have been due to a nonspecific interaction of this protein with the agarose beads or the anti-myc antibody. We also used mitochondria from *T. brucei* expressing TbTim17-2×...
myc as the positive control. Anti-myc antibody pulled down the endogenous TbTim17 along with the ectopically expressed TbTim17-2× myc, showing that they were associated in vivo. An anti-TAO antibody was used as a negative control; no band was detected from the samples immunoprecipitated with this antibody, indicating the specificity of the interactions (Fig. 5B). These results show that ScTim17 is associated with TbTim17 in vivo. Although ScTim23 is present in protein complexes similar to ScTim17 and TbTim17 on BN-polyacrylamide gels, it is apparently more weakly associated with TbTim17 than it is with ScTim17.

**ScTim17 partially complements the growth defect caused by TbTim17 RNAi.** To assess if *S. cerevisiae* Tim17, Tim23, and Tim22 could complement the growth defect in *T. brucei* caused by TbTim17 RNAi, we induced cells of the doubly transfected cell lines ScTim17/TbTim17 KD, ScTim23/TbTim17 KD, and ScTim22/TbTim17 KD using doxycycline. The growth of cells of each cell line in the presence and absence of doxycycline was compared with that of TbTim17-KD cells under the same conditions. Induction of TbTim17 KD inhibited cell growth after 4 days (Fig. 6A), as previously shown (25, 28). Doxycycline treatment also drastically reduced the growth of the ScTim23/TbTim17-KD cells, and cell growth ceased after 4 days (Fig. 6B). Interestingly, when ScTim17 was expressed in TbTim17-KD cells, we found that cell growth was not fully inhibited even within 8 days, although the growth rate was slightly lower over the entire period of analysis (Fig. 6C). These results show that ScTim17 at least partially complemented the growth defect caused by TbTim17 KD. Comparing the growth of ScTim22/TbTim17-KD cells in the presence and absence of doxycycline revealed that expression of ScTim22 failed to restore the growth of *T. brucei*, when TbTim17 was reduced by RNAi (Fig. 6D). Induced cells grew at a lower rate from the beginning, and growth ceased after day 6. These results suggest that neither the yeast Tim23 nor the yeast Tim22 is capable of complementing the TbTim17 function in *T. brucei*. Immunoblot analysis of proteins from isolated mitochondria at day 4 after induction with doxycycline showed that ScTim proteins were present in the mitochondria, while the endogenous levels of TbTim17 were reduced, as expected (Fig. 6E). However, the levels of ScTim proteins were not equivalent. Interestingly, the level of ScTim23 was significantly lower than that of ScTim23 and ScTim22 in TbTim17-KD cells. As mentioned earlier, a similar observation was not seen when we expressed ScTim proteins in the parental *T. brucei* line (Fig. 3A; see also Fig. S2B in the supplemental material). Therefore, this indicates that TbTim17 is possibly required for the import/stability of ScTim17 in *T. brucei*. In spite of the lower level of ScTim17 seen in mitochondria, expression of this protein showed growth complementation for TbTim17 KD. These results thus support the notion that TbTim17 is functionally most similar to ScTim17.

**TbTim17 expression fully complements the growth defect caused by TbTim17 RNAi.** To assess if expression of TbTim17 can reverse the growth defect in *T. brucei* by TbTim17 RNAi, we developed an RNAi cell line in which the double-stranded RNA for the 3′ UTR of the TbTim17 transcript was produced and targeted only the endogenous copy. Thus, the ectopically expressed copy of TbTim17 was unharmed. For this purpose, we first established that this 3′ UTR RNAi could effectively knock down TbTim17. We determined that the TbTim17 protein level was reduced 60 to 70% within 6 days due to induction of RNAi targeted to the 3′ UTR of the *TbTim17* transcript (see Fig. S3A and B in the supplemental material). In comparison to the findings for the RNAi cell line that was previously generated by targeting the coding region of this transcript, we found that this new cell line was relatively less robust (see Fig. S3A and B in the supplemental material). This could be because the 3′ UTR of the TbTim17 transcript could have folded secondary structures, which may inhibit the annealing of some interfering RNAs.

Analysis of the growth kinetics clearly showed that doxycycline significantly reduced cell growth upon induction of the expression of the double-stranded RNA (Fig. 7A). Next, we monitored the growth kinetics of cells of the *T. brucei* cell line that expressed a wild-type copy of TbTim17, in addition to the double-stranded RNA for the 3′ UTR of the TbTim17 transcript. The results showed that upon induction with doxycycline, the cell growth defect caused by TbTim17 KD (by the use of RNAi targeted to the 3′ UTR) was fully complemented by the expression of wild-type TbTim17 (Fig. 7B).

To investigate if the ectopic copy of TbTim17 was expressed in *T. brucei*, cells were harvested at 48 h postinduction and proteins...
FIG 7 Ectopically expressed TbTim17 complements the RNAi growth defect. (A and B) TbTim17-KD (in which TbTim17 was knocked down by the use of RNAi targeted to the 3′ UTR) (A) and TbTim17/TbTim17-KD (B) cells were grown in the presence of doxycycline (induced) or left uninduced. Cell numbers were counted at different time points of growth, and the log cumulative cell numbers were plotted versus the times postinduction (in days). Data represent the log of the mean values from three independent experiments. (C) TbTim17 protein levels in T. brucei 427 29-13 (wild-type) cells, cells with TbTim17 RNAi transfected with a wild-type copy of TbTim17 (TbTim17/TbTim17 KD), and TbTim17-KD cells grown in the absence (−) and presence of (+) doxycycline (Dox). The anti-TbTim17 antibodies were used to detect protein in the immunoblot. TbTim17-myc and endogenous TbTim17 are indicated. Tubulin was used as the loading control. (D) Analysis of the total (lanes T), cytosolic (lanes C), and mitochondrial (lanes M) fractions from TbTim17-KD/TbTim17 cells by immunoblot analysis using anti-TbTim17, anti-TbPP5 and anti-VDAC antibodies. Ten micrograms of protein was loaded per lane.

were analyzed by SDS-PAGE and Western blotting. The parental T. brucei strain (wild type) was grown under the same conditions and used as a control. As expected, there was no change in TbTim17 protein levels in wild-type cells that were either treated or untreated with doxycycline (Fig. 7C). However, two distinct protein bands were seen in TbTim17/TbTim17-KD cells. The lower band (~19 kDa) was the endogenous TbTim17, which was reduced after induction of RNAi with doxycycline (Fig. 7C). The upper band, which is the ectopically expressed TbTim17 with a myc epitope tag at the C terminus, was expressed at the same level in cells grown in the presence or absence of doxycycline. As expected, the TbTim17 protein level was reduced in TbTim17-KD cells grown in the presence of doxycycline.

To confirm the localization of the ectopically expressed TbTim17 in mitochondria, a subcellular fractionation analysis was performed after harvesting TbTim17/TbTim17-KD cells grown in the presence of doxycycline for 48 h (Fig. 7D). T. brucei VDAC and TbPP5 were used as mitochondrial and cytosolic marker proteins, respectively. The ectopically expressed TbTim17-myc was found exclusively in the mitochondrial fraction, similar to the findings for endogenous TbTim17. Together, these results demonstrate that RNAi targeted to the 3′ UTR of the TbTim17 transcript did not affect the ectopic expression of TbTim17-myc. In addition, expression of TbTim17-myc was capable of rescuing cells after TbTim17 KD.

ScTim17 partially complements the defect in mitochondrial protein import caused by depletion of TbTim17. Next, we performed mitochondrial protein import assays to assess if ScTim proteins were functional in T. brucei. For this purpose, we expressed ScTim23, ScTim17, and ScTim22 individually in cells of the MRP2-2× myc/TbTim17-KD cell line. In these cells, both MRP2-2× myc and the double-stranded TbTim17 transcript were expressed after induction with doxycycline. At different time points (24 to 80 h) during induction, the level of MRP2-2× myc in mitochondria was assessed by immunoblot analysis. The MRP2-2× myc protein was expressed in cells of the MRP2-2× myc-transfected T. brucei cell line and was found in the mitochondrial fraction at all time points (Fig. 8A). However, in doubly transfected cells where both TbTim17 double-stranded RNA and MRP2-2× myc were induced simultaneously by doxycycline, we found that the level of this protein was drastically reduced after 48 h (Fig. 8B), showing that reduction of the TbTim17 level by RNAi hampered the import of this nuclear DNA-encoded mitochondrial protein. Next, we investigated whether the expression of any of ScTim proteins aided the import of MRP2-2× myc into mitochondria when TbTim17 was depleted by RNAi. The expression of ScTim23 (Fig. 8C) and ScTim22 (Fig. 8E) failed to help regain the level of MRP2-2× myc in mitochondria. However, expression of ScTim17 partially restored this level in mitochondria when TbTim17 was depleted (Fig. 8D). To assess the relative levels of MRP2 in different mitochondrial samples, the intensity of the MRP2 protein band was quantitated for each sample at 72 h postinduction, its intensity was normalized to the intensity of the corresponding tubulin band, and the results were plotted after setting the level of MRP2 in singly transfected T. brucei cells (T. brucei 29-13 MRP2-2× myc) equal to 100 (Fig. 8F). The results showed that the reduction of MRP2 levels in both ScTim23/TbTim17-KD and ScTim22/TbTim17-KD cells (50 to 60%) was similar to that in cells with TbTim17 KD. However, in ScTim17/TbTim17-KD mitochondria the level of MRP2 was retained at a level >80% of that for the controls. Together, these results show that TbTim17 is functionally closer to ScTim17 than ScTim23. Due to improper sorting, ScTim22 was also not functional in T. brucei.

DISCUSSION

We analyzed the functional similarity of TbTim17 with its homologs in S. cerevisiae by complementation analyses. Our results show that, despite the similarities in their primary and secondary structures, TbTim17 is functionally divergent from its yeast homologs. TbTim17 could not complement the function of any of the three yeast PRAT family members, ScTim17, ScTim23, and ScTim22. Although ScTim23 was properly integrated and assembled into protein complexes like endogenous TbTim17 was, it was nonfunctional in T. brucei. Only ScTim17 partly complemented the deficit of TbTim17 in T. brucei. Although ScTim22 was expressed and targeted to T. brucei mitochondria, it was not inte-
that TbTim17 interacts with TbTim50 (37). A homolog of Tom70 has not been identified in T. brucei. The N-terminal targeting signal in the mitochondrial protein TAO (46) and with a few other trypanosome-specific proteins (28). Together they possibly form the core translocase of about 300 kDa, as observed by BN-PAGE analysis. The core complex likely further associates with other factors to form a larger complex of 1,100 kDa. Data presented here show that ScTim17 can associate with TbTim17 in T. brucei and most likely assembles in the core complex. Thus, it could at least partly complement the downregulation of TbTim17. ScTim23, on the other hand, possesses a longer hydrophilic region at its N terminus, which may hamper its stable association with TbTim17 and/or other components of the 300-kDa TbTim17 protein complex. Further analysis with an N-terminally truncated version of ScTim23 may prove this hypothesis.

Tim23 and Tim17 in yeast are imported into the mitochondrial inner membrane via internal targeting signals located within the transmembrane domains of these proteins (44, 45). ScTim23 and ScTim22 were shown to be recognized by the Tom70 receptor, located on the cytosolic face of the outer mitochondrial membrane (44). Then these proteins cross the outer mitochondrial membrane through the Tom40 channel and are finally inserted into the inner membrane via the TIM22 complex with the assistance of small Tim chaperones in the intermembrane space (IMS) (44). A homolog of Tom70 has not been identified in T. brucei. Despite these deficiencies, the T. brucei mitochondrion is capable of recognizing the targeting signals of ScTim17 and ScTim23. This is because TbTim17 most likely possesses an internal targeting signal similar to the targeting signals of ScTim17 and ScTim23. Besides, we have recently characterized a presequence-like internal targeting signal in the mitochondrial protein TAO (46).

Therefore, T. brucei must possess unidentified receptors for the import of such proteins into mitochondria. ScTim22, however, takes a slightly different route to reach its destination in yeast mitochondria. Although the targeting signal for ScTim22 has not been well characterized, it has been shown that this protein requires the Tom20 receptor, and after translocation through the Tom40 channel, it is also inserted into the mitochondrial inner membrane via the TIM22 complex (47, 48). Recent reports show that the import and assembly of Tim22 in fungi depend on Tim18, a component of the TIM22 translocase, as well as Mia40, an IMS protein (48, 49). Oxidation of a pair of intramolecular sulf-hydryl groups in Tim22 is critical for the import and assembly of this protein into the mitochondrial inner membrane (48, 49). Trypanosomatids do not have a homolog of either Tim18 or Mia40, which could potentially explain why ScTim22 was unable to assemble into the T. brucei mitochondrial inner membrane.

T. brucei also lacks a protein complex similar to TIM22, which in fungi is essential for insertion of Tim23, Tim17, and Tim22. However, the parasite is fully capable of inserting heterologous proteins (Tim23 and Tim17) as well as TbTim17 and many other endogenous internal signal-containing proteins. It can be speculated that TbTim17 plays a role in the import of these proteins. Experiments are needed to establish the role of TbTim17 in the import of MCPs and other internal signal-containing proteins like TbTim17 itself.

Like trypanosomatids, some microsporidian species also pos-
sess a single PRAT protein (50). Microsporidians possess mito-
somes, the relic form of mitochondria, and have been classified in
the group of fungi (50, 51). Mitosomes are double-membrane-
bound organelles which do not have any electron transport system
or an organelle genome. However, mitosomes possess an Fe-S clus-
ter assembly system, which is the only conserved essential
function among all mitochondria and mitochondrion-like orga-
elles. Mitosomes import proteins from the cytosol, and many of
these proteins possess an N-terminal targeting signal with char-
acteristics similar to those of the N-terminal targeting signals of
mitochondrial proteins. Thus, it is likely that the microsporidian
PRAT protein will also be more similar to Tim17 than to Tim23 in
fungi. Parasitic protozoa, such as *Giardia* and *Entamoeba*, also
possess mitosomes (52, 53). Surprisingly, no member of the PRAT
family of proteins has been identified in the genomes of these
protozoa (52, 53). Therefore, it is not clear at this time how mito-
somal matrix proteins cross the inner membrane in this group of
parasites.

In contrast to these organisms, trypanosomatids possess a
fully developed mitochondrion with an electron transport sys-
tem and a mitochondrial membrane potential. Moreover, their
mitochondrial proteins seem to be targeted to the organelle by
either N-terminal or internal signals. Still, a single protein,
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