Downregulation of Mitochondrial Porin Inhibits Cell Growth and Alters Respiratory Phenotype in *Trypanosoma brucei*†‡

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**Trypanosoma brucei** belongs to a group of parasitic protozoa that possess a single tubular mitochondrion with a concatenated structure of mitochondrial DNA known as kinetoplast (30). *T. brucei* is the infectious agent of the disease African trypanosomiasis, which is spread from one mammal to another by the bite of the tsetse fly (53). During transmission from the insect vector to the mammalian host and vice versa, the parasite undergoes various developmental stages accompanied by dramatic changes in mitochondrial activities (15). The bloodstream form that grows in mammalian blood uses glucose as its energy source and suppresses many mitochondrial activities. The bloodstream-form mitochondria lack cytochromes; thus, respiration in this form is solely dependent on the cytochrome-dependent respiratory pathways (CP), in an attempt to compensate for the mitochondrial energy crisis. However, a simultaneous decrease in the substrate-level phosphorylation due to *TbPorin* RNAi caused growth inhibition in the procyclic form. We also found that the expressions of TAO and CP proteins are coordinately regulated in *T. brucei* according to mitochondrial energy demand.

Mitochondrial porin, which is also known as the voltage-dependent anion-selective channel (VDAC), is the most abundant protein in the OM (17, 28). The sizes and the secondary structures of this protein are very similar among different organisms. The VDAC possesses a N-terminal α-helical domain, and the rest of the protein consists of a number of amphiphilic β-strands, which form a barrel-like structure that integrates into the lipid bilayer (16, 17, 28). Recently, the three-dimensional structure of the human VDAC has been elucidated by nuclear magnetic resonance spectroscopy and X-ray crystallography, which showed a β-barrel architecture composed of 19 β-strands and the N-terminal α-helix located horizontally mid-way in the pore (5). *Saccharomyces cerevisiae* and *Neurospora crassa* VDACs also possess 16 to 19 β-strands, similar to the mammalian VDAC (17).

The VDAC exists as different isomeric forms in different species (16, 19). In yeasts, there are two forms: VDAC1 and VDAC2. Only VDAC1 has the channel activity and is abundantly expressed (22, 23). Animals have three isomeric forms: VDAC1 to VDAC3. These isomeric forms showed more than 80% sequence homology among themselves. However, their expression levels and tissue specificities are different (16). Plants also have multiple isomeric forms of the VDAC with various expression levels under different pathological conditions (19). The VDAC plays a crucial role in regulated transport of ADP, ATP, Ca\(^{2+}\), and other metabolites in and out of mitochondria (17, 28, 41). Two ATP-binding sites found at the N- and C-terminal regions in the VDAC are critical for its function (54). Downregulation of VDAC expression disrupts mitochondrial energy production (22, 25). In contrast, overexpression of the VDAC in metazoa...
induces apoptosis, which can be blocked by compounds that inhibit its channel activity (1, 47).

The OM of gram-negative bacteria also consists of various types of porins (24, 32, 40). Based on their structures and functions, they are divided into five groups. OmpA belongs to the small β-barrel integral membrane protein family, which is composed of eight β-strands. It is highly abundant and ubiquitous among most gram-negative bacteria (21). Other types of porins include general porin OmpF, which consists of 16 β-strands; substrate-specific porins, such as LamB or maltoporin, which contains 18 β-strands; receptor-type porin FhuA, the largest β-barrel, with 22 β-strands; and phospholipase A or OMPLA, an integral membrane enzyme containing 12 β-strands (21, 24, 32, 40). The OmpA plays important roles in bacterial conjugation, adhesion, invasion, and immune evasion and also acts as the receptor for several bacteriophages through its surface-exposed loops (44).

Here, we show that the T. brucei mitochondrial porin (TbPorin) possesses a predicted β-barrel structure that has fewer β-strands than other mitochondrial porins but is similar to bacterial OmpA. TbPorin is crucial for mitochondrial energy production via both oxidative and substrate-level phosphorylations. The depletion of TbPorin reduced cell growth of the procyclic form as well as the bloodstream form. Furthermore, it reveals that depletion of mitochondrial ATP level by down-regulation of porin alters the electron flow via TAO and the cytochrome-dependent pathway (CP) as well as the levels of proteins in these pathways.

**MATERIALS AND METHODS**

**Cells.** The procyclic form of the *Trypanosoma brucei* 427 cell line (29-13) resistant to hygromycin and neomycin (G418) and expressing the tetracycline repressor gene (Ter) and T7 RNA polymerase were grown in SDM-79 medium (JRH Biosciences) containing 10% heat-inactivated fetal bovine serum and appropriate antibiotics (hygromycin, 50 μg/ml; G418, 15 μg/ml) (8). Bloodstream-form cells were maintained in HMI-9 medium supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals) and 10% Serum Plus (JRH Biosciences) (43). For the measurement of cell growth, the procyclic-form cells were inoculated at a cell density of 2 × 10^6/ml to 3 × 10^6/ml and the bloodstream-form cells were inoculated at a cell density of 1 × 10^6/ml in medium containing appropriate antibiotics in the presence or absence of doxycycline. Cells were harvested at different time points of growth (6 to 9 days), and the number of cells were counted in a Neubauer hemocytometer. The log of the cumulative cell number was plotted against the time of incubation in culture.

**Comparison of the predicted primary, secondary, and tertiary structures.** Sequence comparison was performed using the ClustalW alignment program (20) in MacVector 10.0. The prediction of the secondary structure of porins was performed using PRED TM (3) and TMPro (36) prediction tools available online. Structure alignment was performed using the iMol software program (4). The prediction of the secondary structure of porins was performed using PRED TM (3) and TMPro (36) prediction tools available online. Structure alignment was performed using the iMol software program (4).

**Generation of an inducible TbPorin RNAi-disrupted cell line and RNA analysis.** To prepare the construct for TbPorin double-stranded RNA expression, the 534 bp fragment of the coding region of the TbPorin gene was PCR amplified from T. brucei genomic DNA by using high-fidelity Pfu polymerase (Stratagene). Sense and antisense primers containing the proper restriction sites at 5’ ends were TbPorin For (5’-GGGATCCCATACAAAGTTGGTGGATGACGC-3’) and TbPorin Rev (5’-AGAACATTGGTTGTACGAAGCGGT-3’). The amplified product was cloned into the BamHI/HindIII sites of a tetracycline-inducible dual-promoter plasmid vector, pET717-177 (50). The construct for TbPorin RNA interference (RNAi) was verified by sequencing. The purified plasmid DNA was linearized by NotI. The linearized plasmid was used for transfection into procyclic-form cells (T. brucei 427-29-13) expressing T7 polymerase and tetracycline repressor proteins according to standard protocols (8). After transfection, the plasmid was integrated into 177-base-pair repeat regions of the minichromosomes in *T. brucei*.

RNA was isolated from the procyclic trypanosomes grown for 4 days with or without doxycycline by using Trizol reagent (Invitrogen) according to the manufacturer’s protocol and was concentrated by 2 M LiCl precipitation. For Northern analysis, RNA was fractionated in formaldehyde-agarose gels (1%) and transferred to nitrocellulose membranes (37). TbPorin and actin gene probes were generated using a random-primer-labeling protocol (Invitrogen) from the TbPorin cDNA clone and the PCR-amplified fragment of the *T. brucei* actin gene. Hybridization was carried out in Rapid-Hyb buffer (Amersham) for 16 h. The membranes were washed at 55°C with 0.1× SSC (150 mM NaCl, 15 mM Na acetate, pH 7.4) containing 0.1% sodium dodecyl sulfate (SDS) and exposed to X-ray film (37). The intensities of TbPorin mRNA bands were quantitated by an imaging densitometer (model GS-700; Bio-Rad) and normalized with the intensity of the corresponding actin mRNA.

**Isolation of mitochondria.** Mitochondria were isolated from the parasite after lysis by nitrogen cavitation in isotonic buffer as described previously (13, 43). The isolated mitochondria were stored at a protein concentration of 10 mg/ml in SME buffer (250 mM sucrose, 20 mM MOPS-KOH, 2 mM EDTA) containing 0.5% glycerol at −70°C. Before they were used, mitochondria were washed twice with 9 volumes of SME buffer to remove glycerol.

**Measurement of ATP.** ATP concentration was measured using an ATP bioluminescence assay kit (Invitrogen) as described previously (38). Mitochondria and mitoplasts were suspended in an ATP assay buffer (20 mM Tris-HCl, pH 7.4, 15 mM KH2PO4, 0.6 mM soroibitol, 10 mM MgSO4, 2.5 mg/ml fatty acid-free bovine

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manufacturer's protocol. Briefly, 10 of the ATP concentration by luciferase ATP assay reagents according to the were centrifuged again, and the resultant supernatants were used for estimation solubilized supernatants were clarified by centrifugation at 100,000 \( \times \) g for 30 min. Supernatants were collected in fresh tubes, immediately subjected to a vortex. Samples were incubated on ice for 30 min and centrifuged at 13,000 rpm for 5 min. Supernatants were collected in fresh tubes, and 20 \( \mu \)l of 1 N KOH was added to each tube to neutralize the acid. Samples were centrifuged again, and the resultant supernatants were used for estimation of the ATP concentration by luciferase ATP assay reagents according to the manufacturer’s protocol. Briefly, 10 \( \mu \)l of the samples were mixed with 40 \( \mu \)l of 0.5 M Tris-acetate, pH 7.75, and 50 \( \mu \)l of luciferase reagent. The fluorescence intensity was measured with a luminometer by using a 1-s delay and an integration time of 1 to 10 s. The ATP concentration for each sample was assayed in triplicate.

**Measurement of oxygen consumption.** Oxygen consumption by whole cells and isolated mitochondria was measured in a closed chamber of 0.5 ml by an oxygen electrode connecting to an amplifier (YSI model 5300) and recorded on graph paper as a trace (49). Procyclic cells were harvested and resuspended in SDM-79 medium containing 10% fetal bovine serum. For each assay, 3 \( \times \) 10^5 cells were used. Salicylhydroxamic acid (SHAM), the inhibitor for alternative oxidase, and antimycin A (2.7 \( \mu \)M) when succinate was used as the substrate. At different time points, samples (100 \( \mu \)l) were treated with 2.5 \( \mu \)l of 60% perchloric acid and 1% digitonin. The protein in each sample was separated by Blue native PAGE (BN-PAGE).

**RESULTS**

**TbPorin possesses a \( \beta \)-barrel structure similar to bacterial OmpA.** We identified by BLAST analysis the gene for mitochondrial porin with duplicate copies (Tb927.2.2510 and Tb927.2.2520) in the *T. brucei* genome database (6) by using *Neurospora crassa* mitochondrial porin as the query. The two copies of TbPorin are identical in their coding sequences and tandemly arranged on chromosome 2 in *T. brucei*. The predicted size of the encoded protein is 29.1 kDa, and the isoelectric pH is 9.6. A ClustalW alignment of this protein with several other mitochondrial porins from fungi and humans showed that TbPorin has 13 to 16% overall identity and 27 to 32% similarity (Fig. 1; see Table S1 in the supplemental material). A BLAST search in the NCBI protein database by using TbPorin as the query showed a strong homology of this protein with six hypothetical proteins in *Trypanosoma cruzi* (64% identity and 74.4% similarity) and the hypothetical protein in each of the *Leishmania* species (e.g., *L. major* [39% identity and 57% similarity], *L. infantum*, and *L. braziliensis*). The BLAST search with TbPorin also showed homology with the porin-like proteins from *Toxoplasma* and *Euglena* spp.

The VDAC has two conserved nucleotide binding sites, one near the N-terminal end and the other near the C-terminal end of the protein. The VDAC has two conserved nucleotide binding sites, one near the N-terminal end and the other near the C-terminal end of the protein. The VDAC has two conserved nucleotide binding sites, one near the N-terminal end and the other near the C-terminal end of the protein. The VDAC has two conserved nucleotide binding sites, one near the N-terminal end and the other near the C-terminal end of the protein. The VDAC has two conserved nucleotide binding sites, one near the N-terminal end and the other near the C-terminal end of the protein.
sesses characteristic sequences similar to those of other mitochondrial porins.

Analysis of the secondary structure using PRED-TMββ prediction tools indicated that TbPorin possesses eight transmembrane β-strands that alternate with nonmembranous loops. Both the N and C termini of TbPorin are predicted to be composed of α-helices (Fig. 1). In contrast, the *Saccharomyces cerevisiae* and human VDAC1s possess 12 and 15 predicted β-strands, respectively. The predicted C-terminal α-helix of the TbPorin is significantly longer than that of yeast and human VDAC1s. The three-dimensional structure analysis using the TMBPro prediction program showed that, similar to yeast and human VDAC1s, the TbPorin protein sequence can be folded into a β-barrel structure (see Fig. S1A in the supplemental material). However, the number of β-strands in TbPorin is lower than those in *S. cerevisiae* and human VDACs. The prediction of an exceptionally long C-terminal tail in TbPorin was also observed using the TMBPro prediction tool. Together, the data suggest that TbPorin forms a β-barrel structure with a diameter smaller than that in the VDACs of other eukaryotes. TbPorin also has both the N- and C-terminal α-helices possibly exposed in the intermembrane space of mitochondria. A similar structure has been found for OmpA, a member of the membrane-integral β-barrel protein family (21). OmpA consists of eight β-strands and a C-terminal globular domain that is not present in other types of porins (21). Superimposing the predicted structures of TbPorin and OmpA by using the iMol software clearly showed a good match (see Fig. S1B in the supplemental material), suggesting that TbPorin is structurally similar to bacterial OmpA.

**TbPorin is a mitochondrial OM protein.** In order to investigate the subcellular location of TbPorin, cell fractionation followed by immunoblot analysis was performed. *T. brucei* procyclic cells were lysed in isotonic buffer. The cytosolic and mitochondrial fractions were separated by differential centrifugation as described previously (13, 43). Analysis of equal amounts of proteins from total lysate and cytosolic and mitochondrial fractions by immunoblotting using specific antibodies revealed that TbPorin is highly enriched in the mitochondrial fraction, similar to other mitochondrial proteins, e.g., Hsp70 and Cyt c1 (Fig. 2A). As expected, the cytosolic protein TBP5 is exclusively present in the cytosolic fraction. These results showed that TbPorin is localized in mitochondria. Alkalai extraction of the isolated mitochondria followed by immunoblot analysis demonstrated that TbPorin is present in the alkalai-resistant membrane pellet, similar to TAO, but a significant proportion of Hsp70, a matrix protein, is found in the soluble fraction (Fig. 2B), suggesting that TbPorin is membrane integrated. A proteinase K treatment of isolated *T. brucei* mitochondria followed by immunoblot analysis of mitochondrial proteins showed that the TbPorin level decreases with an increasing concentration of proteinase K (Fig. 2C). The level of TbPorin is reduced 50% and 70 to 80% at proteinase K concentrations of 50 and 150 μg/ml, respectively. The CPT, a putative *T. brucei* mitochondrial OM protein, is decreased significantly even at 25 and 50 μg/ml of proteinase K. By contrast, the levels of mitochondrial IM proteins Cyt c1 and Tim17 and the matrix-localized protein Hsp70 are essentially protected during proteinase K digestion. Most of these proteins are completely digested by proteinase K when mitochondrial membrane is solubilized with Triton X-100, showing that these proteins are not inherently resistant to proteinase K. These results indicate that TbPorin is localized in the mitochondrial OM.

**TbPorin expression level is developmentally regulated.** Mitochondrial proteins from the procyclic and bloodstream forms were analyzed by Western blotting using antibodies for TbPorin and a few other mitochondrial proteins in *T. brucei*, e.g., AAC, Tim17, TAO, Cyt c1, and Hsp70. The steady-state expression level of the TbPorin protein is six- to sevenfold higher in the procyclic form than in the bloodstream form of *T. brucei* (Fig. 3A). The levels of AAC and Tim17 in the procyclic-form mitochondria are twofold and fourfold higher than these levels in bloodstream-form mitochondria, as reported previously (43). As expected, TAO is more abundant and Cyt c1 is absent in the bloodstream-form mitochondria (15). The levels of Hsp70 are similar between the procyclic- and bloodstream-form mitochondria. The transcript levels of TbPorin in the two developmental stages reflect a pattern similar to that of the

![FIG. 2. Subcellular localization of TbPorin. (A) Immunoblot analysis of subcellular fractions by using TbPorin-, mitochondrial Hsp70 (mHsp70)-, Cyt c1-, and TBP5-specific antibodies as probes. The different fractions were total lysate (T), cytosol (C), and mitochondria (M). Ten-microgram proteins from each fraction were loaded into each lane. (B) Sodium carbonate extraction follows by immunoblot analysis of mitochondrial proteins from the *T. brucei* procyclic form. After Na2CO3 treatment, mitochondria were reisolated by centrifugation. The supernatant (S) and pellet (P) fractions from 10-μg mitochondrial proteins were analyzed using TbPorin-, TAO-, and mitochondrial Hsp70 (mHsp70)-specific antibodies as probes. (C) Proteinase K digestion of mitochondria (10 μg) followed by immunoblot analysis using TbPorin-, CPT-, Cyt c1-, Tim17-, and mitochondrial Hsp70 (mHsp70)-specific antibodies as probes. Concentrations of proteinase K are indicated at the top. Mitochondria samples were treated with Triton X-100 (1%) along with proteinase K (150 μg/ml) as indicated. After digestion, mitochondria were reisolated by centrifugation and electrophoresed as described in the text.]
protein levels. The procyclic form possesses five- to sixfold more TbPorin mRNA than the bloodstream form (Fig. 3B).

**TbPorin is present as monomeric and oligomeric forms in mitochondria.** It has been found that a single molecule of the VDAC is capable of forming a channel (55). However, on mitochondrial membranes, the monomeric form of the VDAC also assembled in dimeric to hexameric or higher oligomeric forms in different species (16, 55). The chemical cross-linking of proteins with EGS in isolated *T. brucei* mitochondria followed by SDS-PAGE and immunoblot analysis showed that TbPorin is present primarily as a monomer and a fraction of the protein appears as dimers. The dimeric form is detected more with increasing concentrations of EGS from 0 to 0.4 mM. The trimeric and tetrameric forms are detected in the procyclic-form mitochondria only at the concentrations of EGS above 0.1 mM. TbPorin is mostly present as a monomer in the bloodstream-form mitochondria and forms a dimer only at or above an EGS concentration of 0.3 mM (Fig. 4). Since the bloodstream form possesses six- to sevenfold less TbPorin than the procyclic-form mitochondria, the TbPorin monomers are possibly more dispersed on the mitochondrial OM of the bloodstream form and thus could not be cross-linked at lower concentrations of EGS. Together, it showed that, similar to VDACs in other organisms, TbPorin is capable of forming multimeric structures on mitochondrial membranes.

**Depletion of TbPorin reduced cell growth in both procyclic and bloodstream forms.** To explore the physiological role of TbPorin, RNAi studies were performed. Induction of the TbPorin double-stranded RNA in the procyclic form reduced its transcript level more than 95% within 2 days (Fig. 5A). The TbPorin protein level was decreased about 50% and 80% at days 4 and 6, respectively (Fig. 5B). Depletion of TbPorin reduced cell growth in the procyclic form. A consistent difference in cell numbers was observed at day 4 and beyond (Fig. 5C). We also performed TbPorin RNAi in the bloodstream form. A reduction in the TbPorin protein level upon induction of the expression of porin double-stranded RNA was accompanied by inhibition of cell growth also in the bloodstream form (Fig. 5D and E). The cell number was significantly reduced at day 5 of doxycycline treatment, and it
became stationary afterwards. These results clearly indicate that TbPorin is critical for mitochondrial activities thus required for normal cell growth in both forms. Recently, Pusnik et al. reported that depletion of the VDAC affects cell growth of the procyclic form only in the absence of glucose (35). Their double-knockout cell lines grew similarly to controls in regular procyclic medium SDM-79. In contrast, our findings showed that a partial depletion of TbPorin reduced cell growth even in the presence of glucose. This discrepancy could be due to mistargeting of our RNAi construct, although it is unlikely. We took a PCR-based approach to demonstrate that the TbPorin RNAi construct is properly targeted at the 177-base-pair repeat regions in the minichromosomes (see Fig. S2 in the supplemental material). Therefore, the differences between our results and those of Pusnik et al. could be due to differences in the constituents of the semisynthetic SDM-79 medium. Also, it could be possible that during the generation of their double-knockout cell line, cells gradually adapted to growing without mitochondrial porin by increasing the activity of other protein channels on the OM. A similar observation has been reported for S. cerevisiae (23).

**TbPorin is crucial for mitochondrial ATP production.** In order to explore the reason for reduced cell growth due to depletion of TbPorin, we compared the energy production capacities of mitochondria isolated from porin KD and control procyclic cells. Different substrates, such as succinate, α-KG, and pyruvate with succinate, were used to compare in organello ATP production via oxidative and substrate-level phosphorylations as described previously (38). Succinate donates electrons to the mitochondrial electron transport chain, thus producing ATP by oxidative phosphorylation (7, 38), whereas α-KG and pyruvate with succinate generate ATP by substrate-level phosphorylation mediated by succinate dehydrogenase and via the acetyl/succinyl coenzyme A transferase cycle, respectively (7, 38). We observed that depletion of TbPorin reduced mitochondrial ATP production by all these substrates about two- to fourfold relative to the control (Fig. 6). In the wild-type procyclic-form mitochondria, ATP production peaked
within 2 to 5 min after the addition of ADP. After reaching the maximum point, ATP production either gradually dropped or stabilized as the substrate was utilized (Fig. 6A and C). As expected, the addition of ADP alone showed a negligible effect on ATP production (data not shown). In contrast to the control, mitochondria isolated from TbPorin KD cells showed very little stimulation of ATP production after the addition of ADP and substrates. In these mitochondria, ATP production was slightly increased at 2 min in the presence of succinate, α-KG, and pyruvate but could not reach the levels found in the control. In the presence of antimycin, ATP production by succinate was inhibited in the control mitochondria as expected, indicating that ATP is produced by oxidative phosphorylation (Fig. 6A). The residual ATP production in porin KD mitochondria was also decreased by antimycin. When mitochondria were pretreated with water to rupture the mitochondrial OM, the ability to produce ATP upon the addition of succinate was significantly increased in porin KD samples (Fig. 6B). This suggested that a reduction of metabolite flux through the OM in porin KD mitochondria is possibly the cause of reduction of its ability to produce ATP. Together, these results showed that depletion of TbPorin reduced both oxidative and substrate-level phosphorylations in mitochondria because the substrates and ADP could not enter through the OM. A similar observation has been made by other investigators (35).

FIG. 6. TbPorin is crucial for mitochondrial energy production. ATP concentrations were measured using a luciferase-based ATP assay kit as described in Materials and Methods. Mitochondria isolated from uninduced control and TbPorin KD cells grown in the presence of doxycycline for 4 days were incubated separately with substrates succinate (A and B), α-KG, and pyruvate with succinate (C). Succinate was added in duplicate samples in the absence or presence of antimycin (2.7 μM). Mitoplasts were prepared from uninduced control and porin KD mitochondria as described in Materials and Methods and incubated with succinate (panel B). The reaction was started with the addition of ADP (55 μM). At different time points (0 to 30 min), aliquots were collected. Proteins were precipitated by 60% perchloric acid, and the supernatants were used for the luciferase assay. Relative light units (RLU) were plotted against time. Each assay was performed in triplicate.
of COIV, Cyt c1, and Tim17 were increased approximately 30%, 10%, and 5%, respectively, in comparison to the uninduced control (Fig. 7A and B). The expression level of the AAC, a major IM protein, was slightly increased due to TbPorin RNAi (Fig. 7A and B). However, the level of the matrix-localized mitochondrial Hsp70 was unaltered, and the same was observed for tubulin. These results showed that TbPorin is possibly not involved in mitochondrial protein biogenesis in general. However, depletion of TbPorin affects differently the levels of components of the cytochrome oxidase and TAO in the procyclic form.

Observing the changes in the levels of COIV and TAO, we became interested in investigating the effect of TbPorin depletion on the levels of mitochondrial respiratory complexes. The membrane protein complexes from TbPorin KD and control mitochondria were solubilized by digitonin, separated by BN-PAGE, and probed with specific antibodies. Mitochondrial porin in other eukaryotes forms an oligomeric complex on the OM (16, 26, 55). We found that TbPorin forms a complex with an apparent mass of 212 kDa (Fig. 8). This complex was abolished in porin KD mitochondria. The same blot was probed with antibodies against *T. brucei* COIV, Cyt c1, and TAO. The antibodies for Cyt c1 and COIV detected complex III and complex IV, respectively, as expected (56). Interestingly, in the TbPorin KD mitochondria, the levels of both complex III and complex IV were increased in comparison to those in mitochondria of the uninduced control cells. The increase was more pronounced for complex IV (Fig. 8). It has been shown previously that during detergent solubilization of mitochondrial proteins, TAO forms an oligomeric complex (14). In the TbPorin KD mitochondria, the level of this TAO oligomer was reduced (Fig. 8), as found for the TAO protein in the previously described experiment (Fig. 7A). The experiment was repeated multiple times, and consistent results were obtained. Altogether, TbPorin KD decreased the level of TAO but in-

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**FIG. 7.** TbPorin RNAi differentially affects the steady-state level of mitochondrial proteins. (A) The TbPorin KD and uninduced control cells were grown for 4 days in the presence of doxycycline, and mitochondria were isolated as described in the text. Mitochondrial proteins (12.5 and 6.25 μg) were analyzed by immunoblot analysis using antibody probes for TbPorin, TAO, Cyt c1, COIV, AAC, Tim17, mitochondrial Hsp70 (mHsp70), and β-tubulin. (B) The intensities of the respective protein bands were quantitated from three independent experiments by using an imaging densitometer as described in Materials and Methods and normalized with the corresponding β-tubulin protein bands. Percent increases and decreases in the intensity in TbPorin KD cells relative to the control were plotted for different proteins.

**FIG. 8.** Depletion of TbPorin increased the level of respiratory complexes III and IV. Mitochondria isolated from the uninduced control (Con) and TbPorin KD cells were solubilized with digitonin (1%). The solubilized supernatants were clarified by centrifugation at 100,000 × g. The samples with increasing amounts of proteins were electrophoresed by BN-PAGE and immunoblotted with antibodies for porin, COIV, Cyt c1, and TAO. Molecular size marker proteins apoferritin dimer (800 kDa), apoferritin monomer (400 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), and bovine serum albumin (66 kDa) were run on the same gel and visualized by Coomassie blue staining. The porin and TAO oligomeric complexes, the bc1 reductase complex, and the cytochrome oxidase complex are indicated on the side of the corresponding blot developed with porin-, TAO-, Cyt c1-, and COIV-specific antibodies. The experiment was repeated more than three times, and consistent results were obtained.
increased complex III and complex IV levels in the procyclic form mitochondria.

Depletion of TbPorin reduced cellular respiration via AP and increased the capacity of CP. The effect of TbPorin depletion on two terminal oxidases led us to determine its effect on cellular respiration. The procyclic-form cells respire both via a usual CP, which is coupled with oxidative phosphorylation, and also through non-proton motive TAO (15, 49). SHAM is the specific inhibitor of TAO, while KCN is the inhibitor of cytochrome oxidase. These two inhibitors were used to estimate the electron flow via the AP and CP as described previously (49). Induction of TbPorin RNAi reduced the electron flow through the AP. After induction with doxycycline for 4 days, inhibition of cellular respiration by SHAM was reduced to 30% in comparison to the uninduced control value (Fig. 9A). This indicates that reduction of TbPorin reduces the engagement of AP, which is not proton motive. In contrast, the percent inhibition of total respiration by KCN was about 80%, and it was increased by 2 to 3% due to TbPorin KD. The overall rates of respiration of the wild-type and TbPorin KD cells were comparable. We also measured the capacities of the AP and CP in both types of cells. The amount of oxygen consumption inhibited by SHAM in the presence of KCN is considered the AP capacity, and similarly, the amount of oxygen consumption inhibited by the specific inhibitor of one pathway in the presence of the inhibitor of the other pathway. The results represent three independent experiments. Student’s t test analysis of different data sets showed that the results are significant with P values less than 0.05. The initial concentration of oxygen in the chamber was 240 μmol/ml at 27°C.

FIG. 9. Depletion of TbPorin alters the respiratory capacity. Oxygen consumption by cells was measured by oxygen electrode and oxygen monitor as described in Materials and Methods. The uninduced control and TbPorin KD cells were grown in the presence of doxycycline and harvested at day 4 for a respiration assay. (A) The inhibition of oxygen consumption by KCN (1 mM) and SHAM (1 mM) is presented as the percent total oxygen consumption by each type of cells. (B) The AP and CP capacities were measured by calculating the amount of oxygen consumption inhibited by the specific inhibitor of one pathway in the presence of the inhibitor of the other pathway. The results represent three independent experiments. Student’s t test analysis of different data sets showed that the results are significant with P values less than 0.05. The initial concentration of oxygen in the chamber was 240 μmol/ml at 27°C.

DISCUSSION

We analyzed the structure and characterized the function of mitochondrial porin in T. brucei. The predicted structure of TbPorin is distinct in comparison to mitochondrial porins from other species. TbPorin depletion reduced cell growth for the procyclic form as well as the bloodstream form. TbPorin is crucial for mitochondrial metabolite transport and thus essential for ATP production in the procyclic form. We also demonstrated that cellular respiration via the AP and CP can be regulated by changes in metabolite flux through TbPorin in the procyclic form.

TbPorin possesses a predicted β-barrel structure. Few structural features in TbPorin are bioinformatically distinct relative to mitochondrial porins from other species. In contrast to the mitochondrial porins with 16 to 19 β-strands from fungi and humans, TbPorin has only 8 predicted β-strands, a number which is similar to the bacterial OM porins OmpA and OmpX (21). The bacterial OM is enriched with various integral β-barrel proteins. The OmpA family proteins, with a barrel structure of eight β-strands, are ubiquitous in most gram-negative bacteria (21). Further analysis of the structure of TbPorin by X-ray crystallography and nuclear magnetic resonance spectroscopy is required to confirm this similarity. Since T. brucei diverged very early during evolution (42), a bacterial porin on trypanosome mitochondria is not unexpected.

We found that TbPorin is localized on the mitochondrial OM as an integral membrane protein. It is known that the VDAC is functionally monomeric; however, it can cluster in tight but regular groups. It exists in a variety of oligomeric states, from 2- to 20-mers (16, 55). The chemical cross-linking of isolated mitochondria from the procyclic form demonstrated that TbPorin is also present in oligomeric states. However, it primarily exists in the monomeric and dimeric forms; the higher-ordered structures, such as trimers and tetramers, are detected only in the procyclic-form mitochondria at higher concentrations of EGS. BN-PAGE analysis of digitonin-solubilized mitochondrial supernatant showed that TbPorin is present in a complex with an apparent molecular mass of 212 kDa. This could be the hexameric or heptameric form of TbPorin. However, the contribution of detergent micelle in this complex needs consideration. It is also possible that the complex is a hetero-oligomer, as VDAC-interacting partners are found in other eukaryotes (16, 22). Thus, the exact oligomeric state in this complex can only be speculated. The steady-state level of TbPorin is reduced about six- to sevenfold in the bloodstream form of T. brucei. In this form, a majority of TbPorin exists in the monomeric state, indicating that its distribution is less clustered and more dispersed on the OM. In the bloodstream form, mitochondrial activities are suppressed (15). Thus, it can be considered that a reduced level of metabolite flux via a reduced level of mitochondrial porin is sufficient to maintain mitochondrial activities in the bloodstream form. The expression levels of several other mitochondrial proteins, excluding TAO, are also reduced in the bloodstream form. TAO is the only terminal oxidase in this form and is crucial for its respiration. Thus, it is greatly upregulated in comparison to that in the procyclic form.

To evaluate the function of TbPorin, we performed RNAi in the procyclic form as well as the bloodstream form. A reduc-
tion in the TbPorin protein level decreased cell growth in both procyclic and bloodstream forms, indicating that porin is required for mitochondrial activities in both developmental stages. We also found that the mitochondrial ATP level is reduced about twofold in the procyclic form due to TbPorin RNAi. Furthermore, the capacity of isolated mitochondria to produce ATP by oxidative and substrate-level phosphorylations is significantly reduced due to depletion of TbPorin. Disruption of the OM of the porin-depleted mitochondria increased ATP production when substrates were provided. Thus, similar to other eukaryote VDACs, TbPorin mediates the major metabolite flux between the mitochondrion and cytosol, which is necessary for production of ATP. The transport of ADP/ATP occurs in a reverse orientation in the bloodstream-form mitochondria, and this process is crucial for maintaining the mitochondrial membrane potential. It is assumed that TbPorin is involved in the transport of nucleotides and substrates also in the bloodstream-form mitochondria. Therefore, depletion of TbPorin reduces cell growth in this form, too.

TbPorin RNAi differentially affects the steady-state level of mitochondrial proteins in the procyclic form. Interestingly, we found that the expression level of TAO is decreased and those of Cyt_c and COIV were increased in mitochondria in which TbPorin was partially depleted by RNAi. There was no change in the transcript level of these proteins in TbPorin KD cells relative to the control (data not shown), indicating that the regulation possibly occurs at the stage of protein synthesis or protein stability. Using BN-PAGE analysis, we separated mitochondrial membrane complexes. We found that the level of complex IV is significantly increased in TbPorin-depleted mitochondria, as seen for its component proteins. These results are consistent and correlated with the levels of the corresponding components. Furthermore, the increase in the level of the mature cytochrome oxidase complex was also correlated with the respiratory activity via the CP. In the procyclic form, the major respiratory flux goes through the CP and 20 to 30% of the activity remains engaged via the AP (49). Respiration via the AP does not produce ATP; thus, during the ATP crisis in the absence of TbPorin, the electron flow was reduced via the AP. The results are consistent among multiple experiments and also correlated with the reduction in the TAO protein level. Simultaneously, the capacity of electron flow via the CP was increased, as it may cause increased ATP production by oxidative phosphorylation. However, cells could not overcome the ATP demand by this alteration, due to a simultaneous collapse of the substrate-level phosphorylation by TbPorin KD. It has been demonstrated previously that the substrate-level phosphorylation is crucial for the survival of the procyclic form (7, 9).

Besides the proteins that are involved in respiration, the expression levels of some other mitochondrial proteins were also altered due to TbPorin RNAi. The level of T. brucei Tim17, the IM protein translocator, was increased due to TbPorin depletion. The reason for this change is not clear at the moment. However, it has been found in S. cerevisiae that depletion of mitochondrial porin (POR 1) increased the level of the protein translocase of the mitochondrial OM complex (Tom) components, possibly to increase OM permeability due to cellular needs (23). Since T. brucei Tom proteins have not yet been characterized, we could not verify the effect of porin depletion on Tom proteins in T. brucei. It can be anticipated that in TbPorin-depleted mitochondria, the T. brucei Tim17 level was increased due to a similar reason, such as compensation of the metabolite flow in and out of mitochondria.

Overall, we found that TbPorin is crucial for mitochondrial activities in both forms. The substrate-level phosphorylation as well as oxidative phosphorylation for cellular energy production in the procyclic form is dependent on continuous metabolite flux via TbPorin. It has been reported that inhibition of substrate-level phosphorylation alone caused inhibition in cell growth in this form (9). Therefore, it is expected that depletion of TbPorin would also affect cell proliferation in a similar manner unless another protein(s) takes over its function in the absence of TbPorin. Thus, our findings properly justified TbPorin function. Besides the effect on cell growth and mitochondrial ATP production due to TbPorin RNAi, we also demonstrated here that regulation of the metabolite flux through TbPorin regulates the electron flow mediated by two terminal oxidases via increasing or decreasing the levels of the component proteins and their assemblies. In other eukaryotes, VDAC activity is known to be regulated by different cellular components under different physiological conditions to modulate the function of mitochondria (16, 28).

Therefore, it is likely that TbPorin plays a crucial role in regulation of the metabolic pattern with changes in nutrients and other environmental factors that occurs in the parasite life cycle. As mitochondrial OM proteins in this organism are poorly characterized, the TbPorin-specific antibody will also serve as a useful marker for the OM. Altogether, this study revealed that TbPorin is critical for mitochondrial activities, although structurally it is closer to a prokaryotic porin.

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