The Single Mitochondrial Porin of *Trypanosoma brucei* is the Main Metabolite Transporter in the Outer Mitochondrial Membrane

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All mitochondria have integral outer membrane proteins with β-barrel structures including the conserved metabolite transporter VDAC (voltage dependent anion channel) and the conserved protein import channel Tom40. Bioinformatic searches of the *Trypanosoma brucei* genome for either VDAC or Tom40 identified a single open reading frame, with sequence analysis suggesting that VDACs and Tom40s are ancestrally related and should be grouped into the same protein family: the mitochondrial porins. The single *T. brucei* mitochondrial porin is essential only under growth conditions that depend on oxidative phosphorylation. Mitochondria isolated from homozygous knockout cells did not produce adenosine-triphosphate (ATP) in response to added substrates, but ATP production was restored by physical disruption of the outer membrane. These results demonstrate that the mitochondrial porin identified in *T. brucei* is the main metabolite channel in the outer membrane and therefore the functional orthologue of VDAC. No distinct Tom40 was identified in *T. brucei*. In addition to mitochondrial proteins, *T. brucei* imports all mitochondrial tRNAs from the cytosol. Isolated mitochondria from the VDAC knockout cells import tRNA as efficiently as wild-type. Thus, unlike the scenario in plants, VDAC is not required for mitochondrial tRNA import in *T. brucei*.

Introduction

Mitochondrial genomes encode a small subset of the proteins needed for mitochondrial biogenesis and function. More than 95% of all mitochondrial proteins are encoded on genes in the nucleus, synthesized in the cytosol and, finally, imported into mitochondria (Neupert and Herrmann 2007; Bolender et al. 2008). Furthermore, because most mitochondrial genomes also lack a number of essential tRNA genes, the translation of mitochondrially encoded proteins requires the import of specific tRNAs from the cytosol into the mitochondrial matrix (Salinas et al. 2008).

Mitochondrial protein import has been intensively investigated, resulting in a detailed knowledge of the mechanism and the machineries of the process (Neupert and Herrmann 2007; Bolender et al. 2008). It requires heteroligomeric protein complexes in the outer and the inner mitochondrial membranes that are termed TOM and TIM complexes respectively. The TOM complex is composed of a number of small integral membrane proteins arranged around the conserved channel subunit Tom40. Proteins pass through Tom40 and access the internal components of the protein import pathway. In the mitochondrial inner membrane, there are two complexes: the TIM23 complex responsible mainly for the import of proteins into the matrix and the TIM22 complex required for the insertion of multispanning inner membrane proteins.

Much less is known about how tRNAs are imported into mitochondria. Factors required for tRNA import into mitochondria have been identified in yeast and plants and in trypanosomatids such as *Trypanosoma brucei* and *Leishmania* (Salinas et al. 2008). Studies in yeast suggest that tRNA import requires the TOM complex (Tarassov et al. 1995), whereas work in plants suggests the voltage dependent anion channel (VDAC) is the channel for tRNA import (Salinas et al. 2006). Both Tom40 and VDAC are channel-forming β-barrel proteins. VDAC is the most abundant protein of the mitochondrial outer membrane and is conserved in all known mitochondria, forming the main channel by which metabolites are transported across the outer mitochondrial membrane (Colombini 2004; Lemasters and Holmuhamedov 2006). A number of components of the inner membrane respiratory complexes were identified as necessary for tRNA transfer into the mitochondrial matrix of *Leishmania* (Bhattacharyya and Adhya 2004). The fact that different factors were found in the different systems is in line with the polyphyletic origin of mitochondrial tRNA import (Schneider and Marechal-Drouard 2000; Salinas et al. 2008). This is in direct contrast to the monophyletic origin of the mitochondrial protein import system (Dolezal et al. 2006).

The parasitic protozoa *T. brucei* represents a deep branching eukaryotic lineage. As is common in many parasitic organisms, it shows several highly derived features, including aspects of mitochondrial biogenesis (Schneider 2001). Recent genome analysis has shown that unlike other eukaryotes, *T. brucei* appears to have a single TIM complex in place of the TIM23 and TIM22 complexes found in other eukaryotes (Schneider et al. 2008). Moreover, unlike in most eukaryotes, the *T. brucei* mitochondrial DNA is lacking any tRNA genes. All tRNAs required for the translation of mitochondrially encoded proteins are imported from the cytosol. *T. brucei* therefore represents an excellent system to study both mitochondrial protein import and tRNA import (Schneider 2001).

Screening protein databases with hidden Markov model (HMM)-based profiles for Tom40- or VDAC-related sequences we found a relationship that ties together all previously characterized and predicted Tom40 homologs with the experimentally validated VDACs from animals, fungi and plants. We suggest the collective term "mitochondrial porin" to describe this superfamily of proteins. A single member of the mitochondrial porin superfamily is found in trypanosomatids. In *T. brucei*, the previously

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uncharacterized open reading frame (ORF) Tb927.2.2510 encodes the mitochondrial porin. Experimental analysis, using molecular phylogenetics, gene disruption and phenotyping, subcellular localization and biochemical analysis showed the protein to be a highly diverged VDAC, termed TbVDAC, responsible for metabolite transport across the outer mitochondrial membrane. Moreover, our results also show that TbVDAC is neither required for mitochondrial tRNA import nor for mitochondrial protein import.

Materials and Methods

Cells

The *T. brucei* cell line 427 was grown at 27 °C in SDM79 supplemented with 5% fetal calf serum (Brun and Schönenberger 1979). The cell line 29–13 was grown under the same conditions in SDM79 supplemented with 15% fetal calf serum, 25 μg/ml hygromycin and 15 μg/ml G-418. For the experiment shown in figure 4 cells were grown in SDM80, a modified SDM79, that lacks glucose and which is supplemented with 10% of dialyzed fetal calf serum as described (Lamour et al. 2005). Practically, cells were first adapted for approximately 1 week for growth in SDM80 supplemented with 4 mM of glucose and were then assayed for growth in the same medium lacking glucose.

Phylogenetic Analysis

The trypanosomatid mitochondrial VDAC sequences were aligned with amino acid sequences of Tom40s and mitochondrial VDACs from public databases aided by presence of common conserved protein domains identified by the Conserved Domain Architecture Retrieval Tool (CDART; Geer et al. 2002). Removal of ambiguously aligned sites resulted in an alignment of 181 characters. Phylogenetic analyses on these alignments were carried out using maximum likelihood, distance and Bayesian analysis. Maximum likelihood trees were inferred using PhyML 2.4.4 (Guindon and Gascuel 2003). In both cases, site-to-site rate variation was modeled on a gamma distribution with eight rate categories and invariable sites. The shape parameter alpha and proportion of invariable sites were estimated by PhyML using the Whelan and Goldman (WAG) substitution matrix. Distances were calculated by Tree-Puzzle 5.2 (Strimmer and von Haeseler 1996) with the WAG substitution matrix, with eight rate categories and invariable sites all estimated from the data. Bootstraps were calculated using puzzleboot (shell script by A. Roger and M. Holder, http://www.tree-puzzle.de) and the same conditions. Trees were inferred using WEIGHBOR 1.0.1a (Bruno et al. 2000). Bayesian analysis was performed using MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001) implementing the covarion model and using the WAG + Γ model. Metropolis-coupled Markov chain Monte Carlo was run from a random starting tree for 1,000,000 generations sampling every 1,000 cycles. Four chains were run simultaneously, three heated and one cold, with the first 200,000 cycles (200 trees) discarded as the burn-in. Node posterior probabilities were determined from the consensus of the remaining 800 trees.

Production of a Homozygous *TbVDAC* Knockout Cell Line

The two allelic *TbVDAC* gene clusters in *T. brucei* 29–13 cell line were replaced by homologous recombination using either the phleomycin (for the 1. allele knockout) or the blasticidine (for the 2. allele knockout) resistance genes. The DNA fragments used for transfection consisted of the resistance genes flanked by the same sequences that flank the *TbVDAC* gene cluster (fig. 3). Transfection, selection with antibiotics, and cloning were done as described (Beverley and Clayton 1993).

Mitochondrial Isolation

Mitochondria and mitoplasts were isolated from approximately 10^{11} cells each of the wild-type and the *TbVDAC* knockout cell line. Isolation of mitochondria was based on nitrogen cavitation-mediated cell lysis in an isotonic buffer followed by Nycodenz gradient centrifugation (Schneider, Charrière et al. 2007). This procedure has been shown to yield mitochondria with an intact outer membrane. Mitoplasts were purified by hypotonic cell lysis and subsequent Percoll gradient centrifugation (Schneider, Charrière et al. 2007). They were shown to have a disrupted outer membrane (Allemann and Schneider 2000). The release of cytochrome c (CYT C) was used to determine the integrity of the outer membrane of the two organellar fractions.

Miscellaneous

RNA isolation, Northern analysis, and digitonin extractions were done as described (Tan et al. 2002). Standard procedures were used for immunoblots and Southern analysis. Adenosine-triphosphate (ATP) production assays using 25 μg of isolated mitochondria or mitoplast per sample were performed following published protocols (Allemann and Schneider 2000; Schneider, Bouzaidi-Tiali et al. 2007). In vitro import of tRNAs into mitochondria was performed as described in Bouzaidi-Tiali et al. (2007). Antibodies against TbVDAC were raised in rabbits using the three keyhole limpet hemocyanin-conjugated peptides YKDYHKDAKDLLTKN, QKWKLESKFKGPKDK, and TVDKSLKPGVLITHS as antigens.

Results

Phylogenetic Analysis of Tom40 and VDAC

No mitochondrial outer membrane protein has been characterized yet in any trypanosomatid species. Thus, due to our long-standing interest in mitochondrial biogenesis, we sought to detect homologs of VDAC and Tom40 in the parasitic protozoa *T. brucei*. Initial Blast searches, using the sequences of Tom40s and VDACs from other organisms, were not sensitive enough to detect orthologues of either protein in the *T. brucei* genome (Hertz-Fowler et al. 2004; Berriman et al. 2005). We turned next to an HMM analysis, previously used to search Genbank for sequences of TOM and TIM orthologues (Dolezal et al.

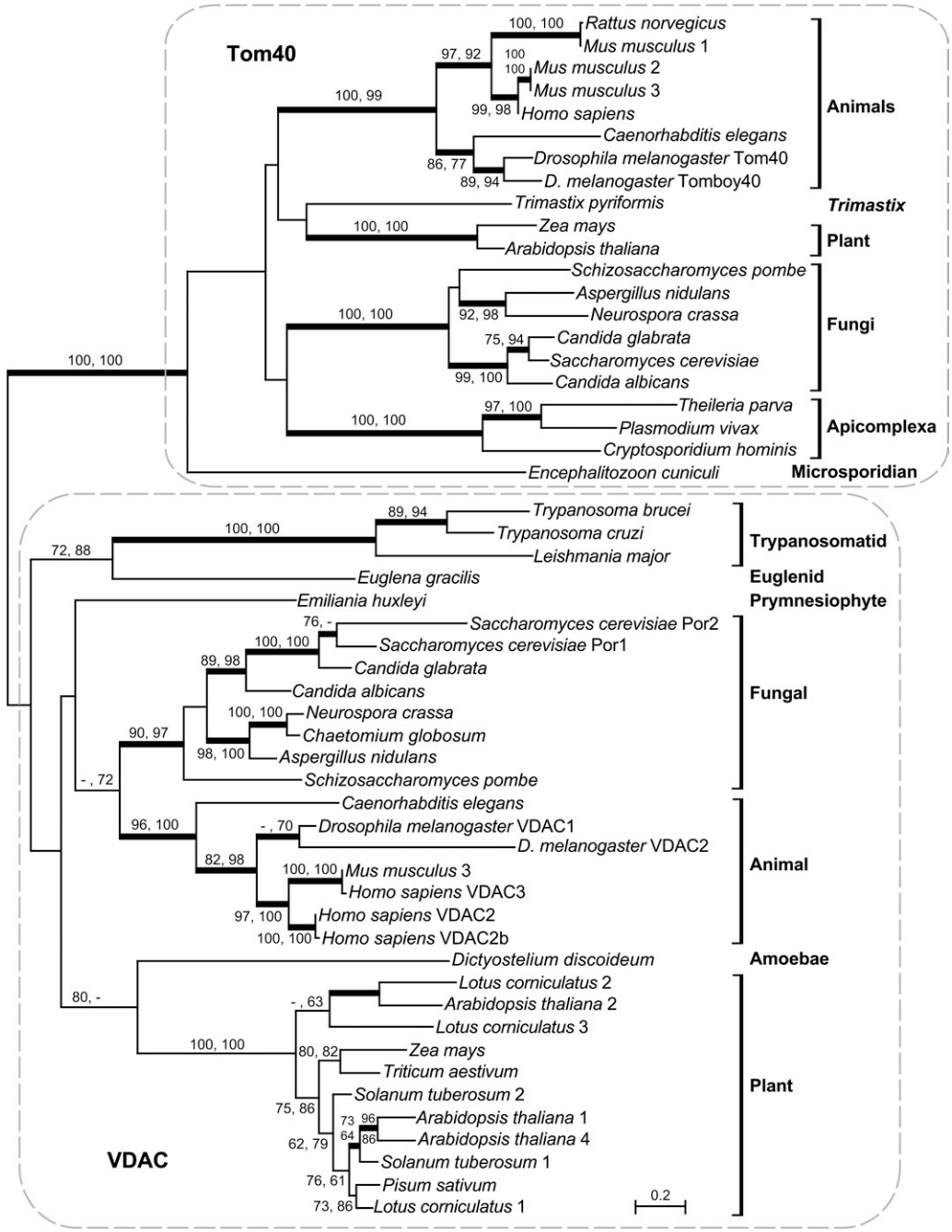

FIG. 1.—Phylogenetic analysis of VDAC and Tom40. Protein maximum likelihood phylogeny of VDAC and Tom40 proteins. Numbers at nodes correspond to bootstrap support over 50% (maximum likelihood left, neighbor joining right), and thickened nodes indicates Bayesian posterior probabilities greater than 0.95. Trypanosomatid proteins resolve within a monophyletic clade of mitochondrial VDACs, distinct from Tom40s.

2006). The Tom40 HMM was built from experimentally validated Tom40 sequences, and yet searches using this HMM retrieved two groups of sequences: 1) a group with the best match to the characteristics of Tom40, including all the previously identified Tom40 sequences and 2) a group of sequences that includes the experimentally validated VDAC from animals, fungi, and plants. This result is mirrored in the "porin-3" family of proteins (PF01459) grouped by CDART analysis (Geer et al.), where the Porin-3 family includes both Tom40s and VDACs. We propose referring collectively to this group as the mitochondrial porins. Searches of the *T. brucei* genome revealed only a single member of the mitochondrial porin family, the protein Tb927.2.2510. The identification of this single protein begs two questions: Is Tb927.2.2510 an orthologue of VDAC or Tom40? Does Tb927.2.2510 play a role in mitochondrial protein and/or tRNA import?

An alignment of Tom40 and VDAC homologues was used to infer a phylogeny of the mitochondrial porins. In this phylogeny, Tom40s were strongly resolved as a distinct clade from VDACs (fig. 1). The major eukaryotic groups represented in the Tom40 clade resolved well (e.g., animals, fungi,

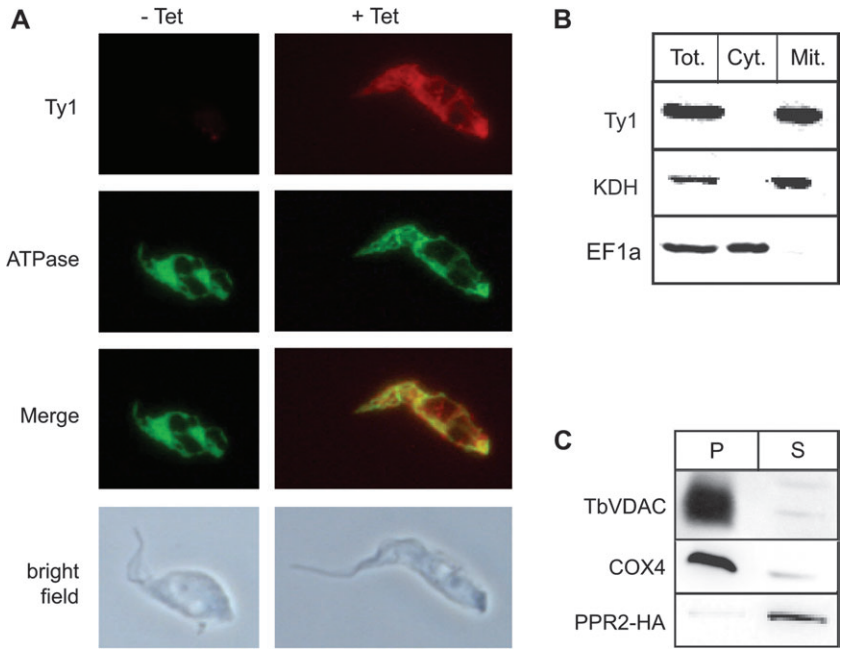

FIG. 2.—Localization of TbVDAC. (*A*) Double immunofluorescence analysis of a *Trypanosoma brucei* cell line expressing TbVDAC carrying the Ty1-tag at its carboxy terminus under the control of the tetracycline-inducible (−Tet, +Tet) procyclin promoter. The cells were stained with a monoclonal antibody recognizing the Ty1-tag (Ty1) and for F1-α subunit of the mitochondrial ATPase serving as a mitochondrial marker. Bar = 10 mm. (*B*) Immunoblot analysis of equal cell equivalents of total cellular (Tot.), crude cytosolic (Cyt.), and crude mitochondrial extracts (Mit.) for the Ty1-tagged TbVDAC (Ty1), KDH served as a mitochondrial marker and elongation factor 1a (EF1a) as a cytosolic marker. (*C*) The panel shows an immunoblot analysis of the pellet (P) and supernatant (S) fractions from carbonate-extracted mitochondrial membranes using antisera against TbVDAC, the integral membrane protein COX 4, and the peripherally membrane-associated hemaglutinin epitope-tagged PPR2 (HA-PPR2). The membrane and matrix fractions correspond to equal cell equivalents. The experiment represents a reprobing of the same blot shown in figure 7 of Pusnik et al. (2007).

plants, and apicomplexans). Significantly, this clade contains all identified Tom40s from diverse eukaryotes, including putative Tom40s from the deep branching excavate *Trimastix* (Hampl et al. 2008) and the highly derived microsporidia. This result provides strong evidence for Tom40 being a very ancient recruitment in the evolution of mitochondria, and that most eukaryotic lineages have maintained a copy of this protein as the core of their TOM complex.

The VDAC clade also resolves the major eukaryotic groups with strong support, as well as some higher order relationships (e.g., sequences from animals show a clear sister relationship to those from fungi). The VDAC clade thus implies that a single copy of this VDAC was inherited with mitochondria in most major groups and that gene duplication has occurred within many of the major groups of eukaryotes. The trypanosomatid proteins resolve within the VDAC clade, distinct from the Tom40 sequences. The three trypanosomatid proteins (from *T. brucei*, *Trypanosoma cruzi*, and *Leishmania major*) group strongly together, and group with moderate support to the euglenid VDAC, consistent with euglenids being the sister group to trypanosomatids. Thus, although these trypanosomatid proteins are related to Tom40s, they are apparently derived from mitochondrial porins of the VDAC group.

Intracellular Localization of TbVDAC

Is the mitochondrial porin Tb927.2.2510 indeed located in mitochondria? We prepared a transgenic cell line allowing inducible expression of a carboxy-terminally tagged version of the protein. As a tag, we used the 10 amino acid long Ty1-peptide (Bastin et al. 1996). Immunofluorescence analysis using anti-Ty1 antibodies showed a tetracycline-inducible staining identical to the one seen with the F1-α subunit of the mitochondrial F_1F_0-ATP synthase (ATPase; fig. 2*A*). Immunoblot analysis of these fractions showed that Tb927.2.2510 is recovered in the pellet together with the mitochondrial marker (fig. 2*B*). This result is consistent with the immunofluorescence analysis and shows that Tb927.2.2510 is located exclusively in mitochondria.

Even though β-barrel proteins do not contain extended hydrophobic transmembrane spans, they are integral membrane proteins. Carbonate extraction of mitochondrial membranes shows that Tb927.2.2510 together with the integral membrane protein cytochrome oxidase subunit 4 (COX 4) is exclusively recovered in the pellet, whereas the peripherally membrane-associated PPR2 protein (Pusnik et al. 2007) partitions in the supernatant fraction. Thus, as expected for a mitochondrial porin, Tb927.2.2510 is an integral membrane protein.

A Homozygous *TbVDAC* Knockout Cell Line

To elucidate whether the mitochondrial porin is essential, and to functionally analyze the protein, we produced cell lines lacking it. In a first approach, we established a cell line allowing inducible, RNAi-mediated ablation of the protein. However, although we were able to efficiently

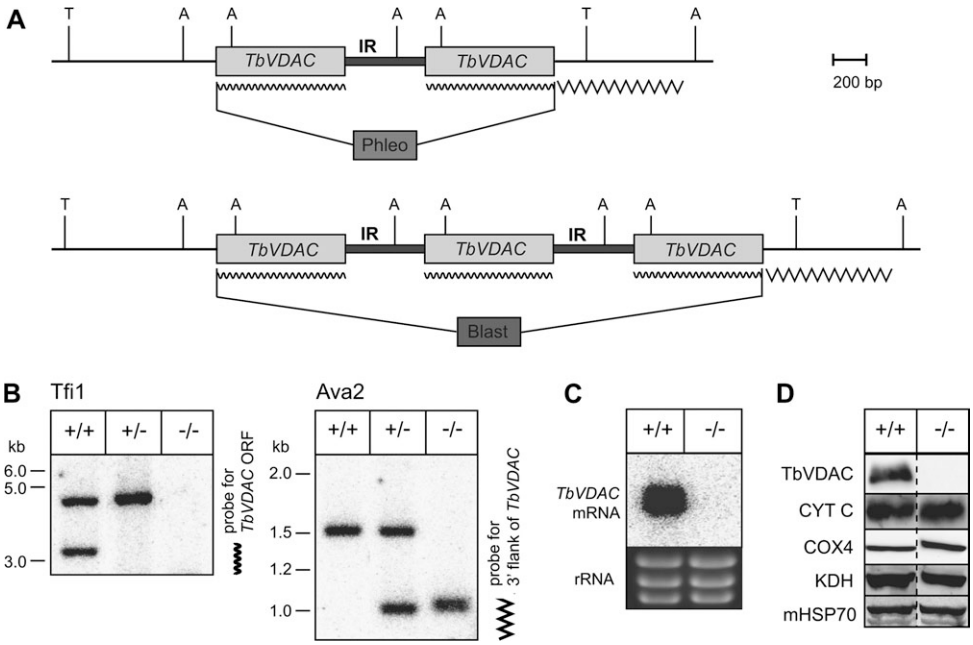

FIG. 3.—Homozygous deletion of the *TbVDAC* locus. (*A*) Schematic drawing of the wild-type *TbVDAC* loci and the situation after homologous recombination leading to replacement of the two loci by phleomycin and blasticidin resistance genes (scale indicated). Restriction sites for TfiI (T), AvaII (A) as well as the intergenic region (IR) are indicated. Jagged lines mark the two probes used in the Southern analysis: They hybridize to the *TbVDAC* ORF and the *TbVDAC* 3′-flanking region, respectively. (*B*) Southern analysis of genomic DNA isolated from the parental cell line (+/+), the single (+/−), and the double knockout cell lines (−/−). The restriction enzymes and hybridization probes are indicated. (*C*) Northern blot of total RNA isolated from the parental cell line (+/+) and the double knockout cells (−/−) hybridized with the probe specific for the *TbVDAC* ORF. Bottom panel shows an ethidium bromide stain of the rRNAs as a loading control. (*D*) Immunoblot of 5 μg of total mitochondrial proteins from the parental cell line (+/+) and the double knockout cells (−/−). Antisera against the following proteins were used: TbVDAC, CYT C, COX4, KDH, and mHSP70.

downregulate the TbVDAC mRNA, this did not affect growth of insect-stage trypanosomes (data not shown). Because RNAi cannot be relied on to deplete protein levels completely, negative data can be misleading. We therefore constructed a *TbVDAC* double knockout cell line. According to the genome sequence, the *TbVDAC* gene is present in two tandemly repeated copies per haploid chromosome. Southern analysis revealed that in our strain, there is a difference between the two alleles in that one contains two and the other three tandemly arranged copies of the *TbVDAC* gene, respectively (fig. 3*A*). In the double knockout cell line, the two *TbVDAC* genes of one allele were replaced by the phleomycin resistance gene and the three remaining copies of the other allele by the blasticidin resistance gene (fig. 3*A*). Consequently, in the double knockout cell line, neither the *TbVDAC* genes nor their corresponding mRNAs or the TbVDAC protein itself can be detected by Southern, Northern, or Immunoblot analysis, respectively (fig. 3*B–D*).

Growth Phenotype of TbVDAC-Lacking Cells

The fact that we could recover a viable double knockout cell line demonstrates that the mitochondrial porin is not required for growth in SDM79, the standard growth medium for procyclic *T. brucei* (Brun and Schönenberger 1979). Figure 4*A* shows that there is no difference in growth rate in SDM79 medium between the $TbVDAC^{-/-}$ cells and the $TbVDAC^{+/+}$ parental cell line from which they were derived. This provides strong evidence that TbVDAC does not function as the core protein import channel because mitochondrial protein import is essential under all growth conditions.

The energy metabolism in procyclic *T. brucei* is complicated, with the presence or absence of glucose determining the means by which parasites produce ATP. For cultured cells, the main carbon source used in SDM79 medium is glucose, and cells grown in SDM79 medium produce ATP mainly by substrate-level phosphorylation (Bochud-Allemann and Schneider 2002; Besteiro et al. 2005). The key enzymes responsible for ATP production under these conditions are the cytosolic phosphoglycerate kinase and pyruvate kinase and the mitochondrial succinyl-CoA synthetase (Bochud-Allemann and Schneider 2002) in the acetate:succinate CoA transferase cycle (Riviere et al. 2004). As an alternative, cells can be grown in SDM80 (Lamour et al. 2005): This glucose-lacking medium reflects more closely the growth conditions found in the hemolymph of the Tsetse fly, where proline is the major carbon source available for ATP production by oxidative phosphorylation. Thus, although oxidative phosphorylation is optional in SDM79 medium, it becomes essential when cells are grown in SDM80 medium that lacks glucose. We compared growth of the $TbVDAC^{-/-}$ cells with the parental cell line in SDM80 medium and, as a control, in the same medium supplemented with glucose. Figure 4*B* shows that whereas the $TbVDAC^{-/-}$ cells fail to grow in the absence of glucose and start to die after 2 days, they grow as well as the parental cell line in SDM80-containing glucose. The parental cell line, however, grows only marginally slower in the absence of glucose. These results suggest that the single mitochondrial porin

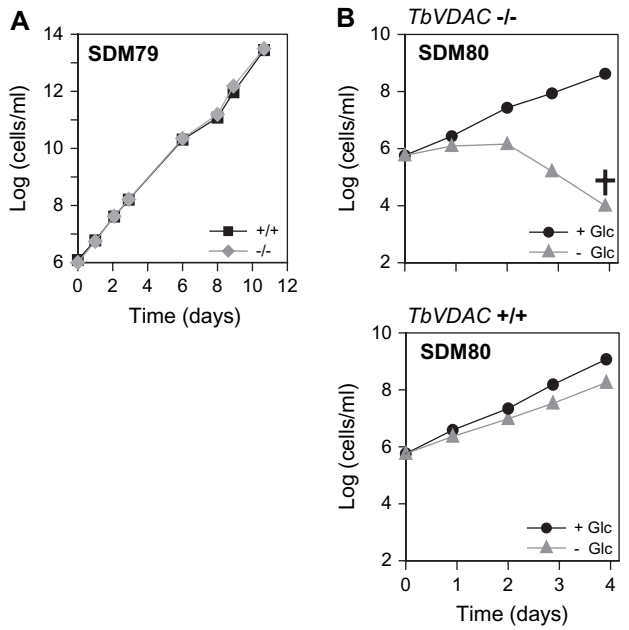

FIG. 4.—TbVDAC is required for growth and survival in glucose-free culture medium. (*A*) Representative growth curve of the *TbVDAC*$^{-/-}$ cells (−/−) (and the corresponding parental cell line 29–13 (+/+) in standard culture medium SDM79. (*B*) Top graph; representative growth curve of the *TbVDAC*$^{-/-}$ cells tested in SDM80 medium supplemented with glucose (black circles) or lacking glucose (gray triangles). Bottom graph: same as top graph, but results are for the parental cell line 29–13.

of *T. brucei* is required to support efficient oxidative phosphorylation.

The phylogenetic analysis (fig. 1), the localization (fig. 2), and the fact that the trypanosomal mitochondrial porin is essential for oxidative phosphorylation but not for life in general (fig. 4) suggest that it functions as a VDAC that forms a metabolite channel across the outer mitochondrial membrane and not as a Tom40. This is further supported by figure 3*D* showing that isolated mitochondria of *TbVDAC*$^{+/+}$ and *TbVDAC*$^{-/-}$ cells contain the same amounts of CYT C (an intermembrane space protein), of COX 4 (an inner membrane protein) as well as of α-ketoglutarate dehydrogenase (KDH) and mitochondrial heat shock protein 70 (mHSP70; two matrix proteins), all of which are expected to be imported across the TOM channel.

In organello ATP Production in TbVDAC-Lacking Cells

In order to provide direct evidence for a role of the mitochondrial porin in metabolite transport, we performed ATP production assays in isolated mitochondria. Mitochondria were isolated from wild-type or *TbVDAC*$^{-/-}$ cells and incubated with substrate and adenosine-diphosphate (ADP), and the resulting ATP production was measured by a coupled luciferase assay (Allemann and Schneider 2000; Bochud-Allemann and Schneider 2002; Schneider, Bouzaidi-Tiali et al. 2007). In these assays, isolated mitochondria become depleted of nucleotides and substrates, so that in organello ATP production requires the addition of ADP and substrates. These substances require a metabolite channel in the outer membrane and specific carrier proteins in the inner mitochondrial membranes in order to contribute to metabolism in the matrix.

Succinate-induced ATP production could be measured in mitochondria from the parental cell line but was abolished in mitochondria isolated from the *TbVDAC*$^{-/-}$ cell line (fig. 5). The same was observed when α-ketoglutarate was used as a substrate (fig. 5*B*, bottom panels). Succinate induces oxidative phosphorylation, whereas α-ketoglutarate induces substrate-level phosphorylation in the citric acid cycle.

Proof that mitochondrial and not cytosolic ATP production was measured in the experiment was provided by the fact that succinate-induced oxidative phoshporylation was sensitive to antimycin and that α-ketoglutarate-induced substrate-level phosphorylation was inhibited by atractyloside. Antimycin is a specific inhibitor of complex III and atractyloside prevents the transport of ADP across the inner mitochondrial membrane.

If the lack of ATP production in the *TbVDAC* knockout cell line is due to the inability of ADP and other substrates to cross the outer mitochondrial membrane, it should be reversed after disruption of the outer membrane. In order to test this, we performed the ATP production assays in mitoplasts (which lack the outer membrane) obtained from the *TbVDAC* knockout cells and the parental cell line. The state of the outer membrane in the two organellar fractions was checked by an antiserum against CYT C. This intermembrane-space-localized protein is rapidly lost if the outer membrane becomes disrupted. Figure 5*C* shows that whereas the same amounts of the matrix protein KDH are found in mitochondria and in mitoplasts, CYT C is essentially absent in mitoplasts confirming the disruption of the outer membrane. The result shows that mitoplasts from *TbVDAC* knockout cells, in contrast to mitochondria with an intact outer membrane, are able to produce ATP in response to both substrates (fig. 5*B*, right panels) with an efficiency comparable to that of both intact mitochondria or of mitoplasts of the parental cell line (fig. 5*A*). These results are in line with the model that the identified mitochondrial porin is the major metabolite channel of the mitochondrial outer membrane, indicating that it is a functional homologue of VDACs in other organisms.

TbVDAC Does Not Promote tRNA Import

Trypanosomatids lack mitochondrial tRNA genes and therefore have to import all mitochondrial tRNAs from the cytosol (Schneider 2001). In plants, the mitochondrial porin VDAC is one of the components of the tRNA import machinery (Salinas et al. 2006). To determine if this is the case in *T. brucei*, we isolated mitochondria from the *TbVDAC* knockout and the parental cell line for tRNA import experiments. Import was defined as ATP-dependent RNase protection of the added tRNA substrate (fig.6*A*, top panel). Quantitative analysis of three independent experiments showed that mitochondria of the *TbVDAC*$^{-/-}$ cells, as well as the parental cell line, were able to import tRNAs with equal efficiency (fig. 6*B*), with import in all cases being characteristically ATP dependent. Figure 6*C* shows that the CYT C is present in equal amounts in both mitochondrial fractions, indicating that the outer membrane remained intact during isolation. These results show that TbVDAC,

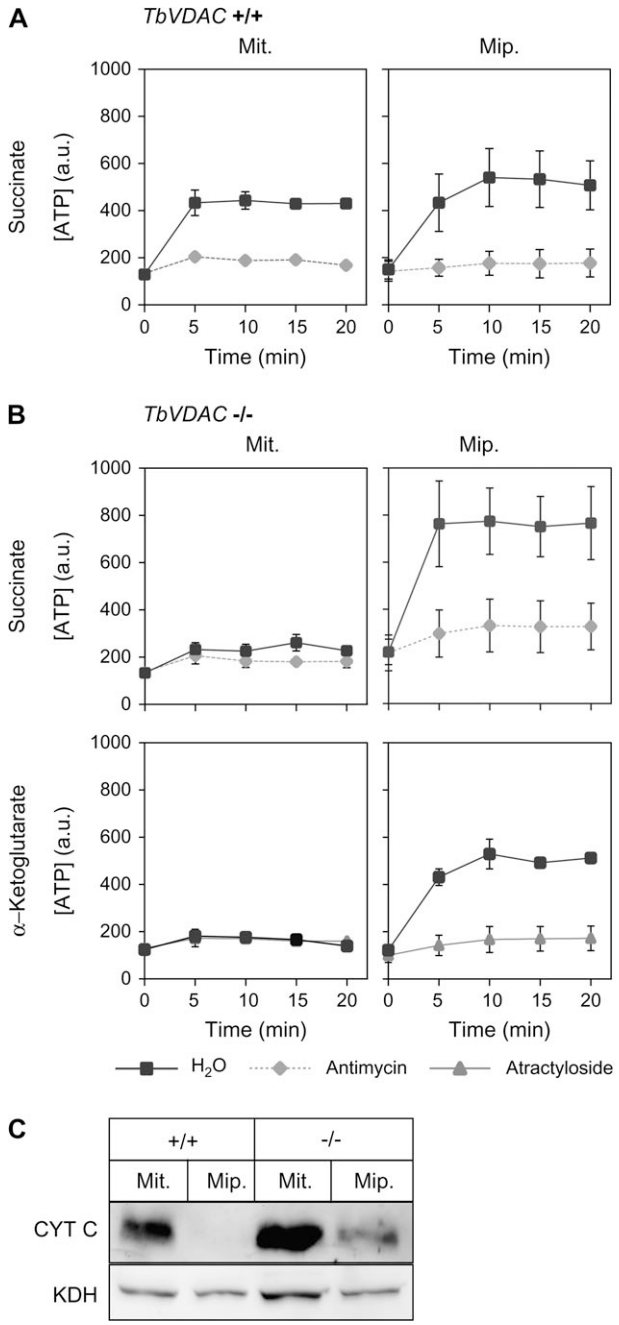

FIG. 5.—ATP production in isolated mitochondria and mitoplasts of $TbVDAC^{+/+}$ and $TbVDAC^{-/-}$ cells. (*A*) Time course of ATP production in response to succinate-induced oxidative phosphorylation measured in isolated mitochondria (Mit.) and in mitoplasts (Mip.) of the parental cell line $TbVDAC^{+/+}$. ATP production is considered to be due to mitochondrial oxidative phosphorylation if it can be inhibited by antimycin. (*B*) Same as (*A*) but ATP production was measured in mitochondria and mitoplasts isolated from $TbVDAC^{-/-}$ cells. Top panels: succinate-induced antimycin-sensitive oxidative phosphorylation. Bottom panels: α-ketoglutarate-induced atractyloside-sensitive substrate-level phosporylation. (*C*) Immunoblots of mitochondria and mitoplasts from $TbVDAC^{-/-}$ cells (−/−) and from the corresponding $TbVDAC^{+/+}$ cell line (+/+) using antisera against the intermembrane-space-localized CYT C or the matrix localized KDH, respectively. Graphs depict the mean and standard errors from three independent experiments.

unlike its countepart in plants, is not required for mitochondrial tRNA import.

Discussion

We have identified and characterized ORF Tb927.2.2510 as the VDAC orthologue in *T. brucei*. It represents the first mitochondrial outer membrane protein characterized in this organism. The assignment is based on 1) the substrate-induced ATP production deficiency of TbVDAC-lacking mitochondria that can be overcome by disruption of the outer membrane and 2) on the clustering of the trypanosomal protein to the mitochondrial VDAC clade in a phylogenetic analysis. The trypanosomes are highly diverged from other eukaryotic groups and highly evolved organisms given the extreme constraints of a parasitic lifestyle. This divergence is reflected in the sequence of TbVDAC, which in the phylogenetic analysis defines the most widely diverged branch of the VDAC clade.

Most eukaryotes, including mammals, insects, plants, and yeast have several VDAC isoforms displaying different properties and probably functions (Blachly-Dyson et al. 1997; Komarov et al. 2004). Unexpectedly, *T. brucei* has a single VDAC isotype only. Because both yeast and *T. brucei* are unicellular organisms, it is interesting to compare our results with the situation in yeast. The two yeast VDACs, Por1 and Por2, are 49% identical and have been characterized in detail. Por1 functions as a metabolite carrier, whereas Por2 on its own is not able to form membrane channels. Simultaneous deletion of both yeast VDACs impaired growth on nonfermentable carbon sources at elevated temperature. However, it only marginally affected growth on these media at normal temperature (Blachly-Dyson et al. 1997). This indicates that in yeast, some level of oxidative phosphorylation can be maintained even in the absence of VDACs and therefore that alternative pathways for transport of metabolites across the outer membrane must exist. The single VDAC in *T. brucei* is the major metabolite channel in the outer membrane because in its absence isolated mitochondria were not able to produce ATP. Growth of trypanosomes in proline-containing medium lacking glucose can be compared with growth of yeast on nonfermentable carbon sources. In both situations, the cells are forced to produce ATP by oxidative phosphorylation exclusively. However, whereas a VDAC-lacking yeast strain was able to grow on nonfermentable carbon sources (Blachly-Dyson et al. 1997), VDAC-lacking *T. brucei* cells could not grow in the absence of glucose (fig. 4). Thus, it appears that in *T. brucei*, unlike in yeast, efficient oxidative phosphorylation cannot be maintained in the absence of VDAC. Nevertheless, it is clear that also in *T. brucei* an alternative pathway for outer membrane metabolite transport must exist for the following reasons: 1) Knockdown of succinyl-CoA synthetase was lethal even in the presence of glucose, indicating that mitochondrial substrate-level phosphorylation is essential (Bochud-Allemann and Schneider 2002). 2) $TbVDAC^{-/-}$ cells still replicate their mitochondria (in the presence of glucose), so that there must be a pathway for nucleotide import to replicate the mitochondrial DNA. The fact that $TbVDAC^{-/-}$ cells grow as efficiently as wild type in glucose-containing medium therefore indicates that metabolites

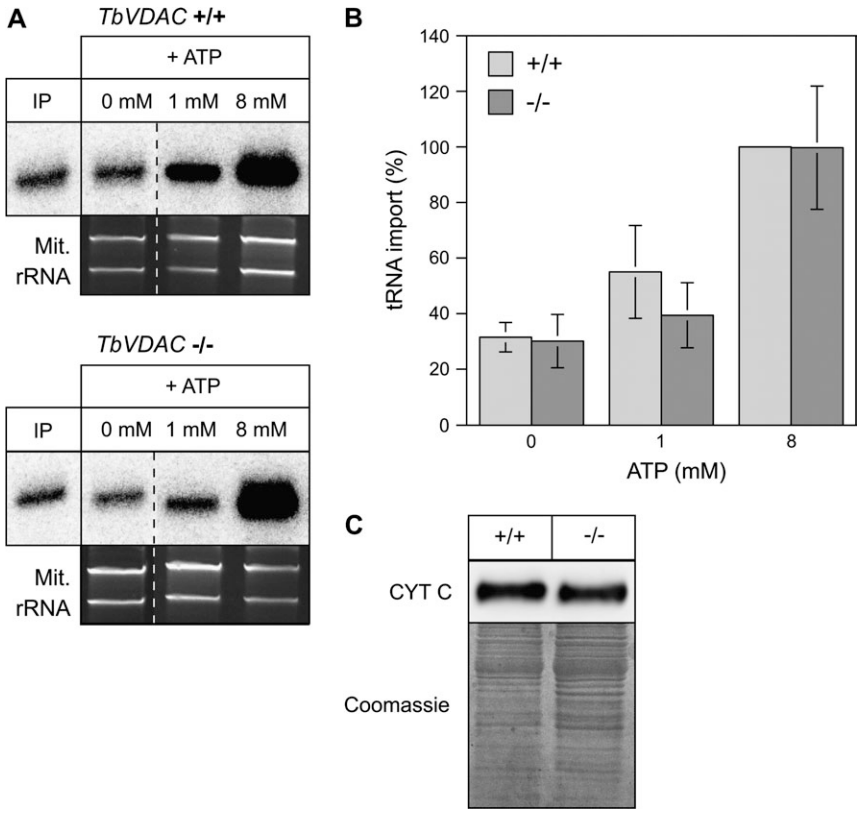

FIG. 6.—tRNA import into isolated mitochondria. (*A*) Top panel, import of in vitro transcribed yeast $tRNA^{Phe}$ into isolated mitochondria from the $TbVDAC^{+/+}$ cell line in the absence and the presence of 1 and 8 mM ATP. The input lanes (IP) depict 1% of the added substrate. All import reactions were treated with micrococcus nuclease. Imported $tRNA^{Phe}$ is detected by Northern blot and specific oligonucleotide hybridization. The ethidium bromide stained panel shows the two mitochondrial rRNAs and serves as loading control. Bottom panel, same as top panel but import was tested using mitochondria from $TbVDAC^{-/-}$ cells. (*B*) Quantification of triplicate import experiments shown in (*A*). Import into mitochondria of the parental cell line at 8 mM ATP was set to 100%. Standard errors are indicated. (*C*) Immunblot analysis of isolated mitochondria from $TbVDAC^{-/-}$ (−/−) cells and from the $TbVDAC^{+/+}$ (+/+) cell line for the presence of the intermembrane space marker CYT C to show the integrity of the outer membrane. A Coomassie stain of the blotted gel serves as a loading control.

can cross the outer membrane in quantities sufficient to support mitochondrial substrate level phosphorylation and replication of mitochondrial DNA.

How can metabolites cross the outer membrane in the absence of VDAC? It has been suggested for yeast that metabolites may also use the channel of the TOM complex whose function is to import proteins (Kmita et al. 2004). In trypanosomes, such a scenario is complicated by the apparent absence of a Tom40 orthologue (discussed below). However, an orthologue of Sam50, a highly conserved β-barrel structured protein that functions in the insertion of β-barrel proteins into the outer mitochondrial membrane, does exist (Wiedemann et al. 2003) (data not shown). Thus, it is conceivable that in the absence of TbVDAC, the trypanosomal Sam50 would provide a channel for low efficiency transport of metabolites across the mitochondrial outer membrane.

The outer membrane of all mitochondria studied so far contains at least three β-barrel proteins: Sam50, Tom40, and VDAC. Sam50 is derived from the bacterial Omp85 (Dolezal et al. 2006). Because they are β-barrel proteins, it is likely that Tom40 and VDAC were also derived from bacterial outer membrane proteins, and given the relatedness we discovered, it is likely that they derive from a common ancestor. However, it has not yet been possible to identify a bacterial protein with homology to either the Tom40 or VDAC subfamily of mitochondrial porins.

Insect-stage *T. brucei* has a highly developed mitochondrion and depends on oxidative phosphorylation for energy production (Schneider 2001). As in most other eukaryotes, the mitochondrial genome encodes only a few proteins, indicating that hundreds of proteins must be transported across the mitochondrial outer membrane. The absence of a Tom40 homologue in *T. brucei* is therefore surprising and raises the question of how proteins are translocated across the outer membrane? We recognize three scenarios of how this might be achieved:

1. A Tom40 orthologue was never there in the first place. This seems unlikely in the light of the fact that except in trypanosomatids, a Tom40 orthologue has been identified in essentially all eukaryotes (Maćasev et al. 2004; Cavalier-Smith 2006; Dolezal et al. 2006).
2. A Tom40 orthologue was there initially, but trypanosomatids have responded to evolutionary pressure to promote a different form of translocase to take over, allowing the Tom40 orthologue to be lost. This remains a reasonable prospect; there are many peculiarities of the trypanosome mitochondrion, such as a massive requirement for tRNA import, RNA editing, and a uniquely

structured mitochondrial genome (Schneider 2001). Moreover, the short more defined targeting sequences that are found in mitochondrially imported proteins of *T. brucei* (Hauser et al. 1996; Häusler et al. 1997) could have driven the development of a new form of translocase.

3. A Tom40 orthologue does exist, but its sequence has diverged so far as to be unrecognizable to current search protocols.

Deciding between these three scenarios is likely to require a direct biochemical approach to identify the apparatus responsible for outer membrane translocation of proteins in *T. brucei*. Whatever result is obtained in such a study, it will be of relevance for both understanding mitochondrial biogenesis in *T. brucei* as well as in the field of mitochondrial protein import in general.

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