Role of Tob55 on mitochondrial protein biogenesis in *Trypanosoma brucei*

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A B S T R A C T

Mitochondrial outer membrane (MOM) proteins in parasitic protozoa like *Trypanosoma brucei* are poorly characterized. In fungi and higher eukaryotes, Tob55 is responsible for the assembly of β-barrel proteins in the MOM. Here we show that *T. brucei* Tob55 (TbTob55) has considerable similarity in its primary and secondary structure to Tob55 from other species. TbTob55 is localized in *T. brucei* MOM and is essential for procyclic cell survival. Induction of Tob55 RNAi decreased the level of the voltage-dependent anion channel (VDAC) within 48 h. Although the primary effect is on VDAC, induction of TbTob55 RNAi for 96 h or more also decreased the levels of other nucleus encoded mitochondrial proteins. In addition, the mitochondrial membrane potential was reduced at this later time point possibly due to a reduction in the level of the proteins involved in oxidative phosphorylation. However, mitochondrial structure was not altered due to depletion of Tob55. *In vitro* protein import of VDAC into mitochondria with a 50–60% reduction of TbTob55 was reduced about 40% in comparison to uninduced control. In addition, the import of presequence-containing proteins such as, cytochrome oxidase subunit 4 (COIV) and trypanosome alternative oxidase (TAO) was affected by about 20% under this condition. Depletion of VDAC levels by RNAi did not affect the import of either COIV or TAO. Furthermore, TbTob55 over expression increased the steady state level of VDAC as well as the level of the assembled protein complex of VDAC, suggesting that similar to other eukaryotes TbTob55 is involved in assembly of MOM β-barrel proteins and plays an indirect role in the biogenesis of mitochondrial preproteins destined for the mitochondrial inner membrane.

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1. Introduction

Trypanosoma brucei, a haemoflagelated parasitic protozoan, causes a fatal disease in humans and domestic animals known as African trypanosomiasis [1]. *T. brucei* belongs to a group of earliest eukaryotes, which diverge very early during evolution [2]. The parasite possesses a single tubular mitochondrion with many unique characteristics [3]. In spite of various complexities, the parasite's mitochondrial genome encodes a handful of mitochondrial proteins. Thus, similar to other eukaryotes, a vast majority of mitochondrial proteins are nuclear encoded and are imported after their synthesis in cytosol [3,4]. However, the mitochondrial protein import machinery in *T. brucei* has been poorly characterized. Recently a homolog of Tim17, a component of the translocase of mitochondrial inner membrane (TIM) in other eukaryotes, has been identified and characterized in *T. brucei* [5,6]. Searches in the *T. brucei* genome database found homologs for a few small Tims

of the intermembrane space (IMS) [5]. However, none of the subunits of the translocase of mitochondrial outer membrane (TOM) have been identified in *T. brucei*. Thus, how proteins cross the mitochondrial outer membrane (MOM) in *T. brucei* remains enigmatic.

In other eukaryotes, MOM possesses several β-barrel proteins [7,8]. These include Tom40 [9,10], VDAC (voltage-dependent anion channel, also called porin) [11,12], Tob55 (topogenesis of β-barrel protein; also called Sam50) [13,14], and Mdm10 and Mmm2 (mitochondrial morphology proteins) [8,15]. Among these, Tom40 and Tob55 are crucial for biogenesis of nuclear encoded mitochondrial proteins [13,14,10]. Tom40 is the major component of the TOM complex and responsible for import of virtually all types of mitochondrial proteins [10].

Tob55 is needed for biogenesis of mitochondrial β-barrel proteins such as VDAC and Tom40 [13,14]. The TOB complex in fungi possesses two more proteins Sam35/Tob38 and Sam37/Mas37. The Tob55 and Tob38 are essential OM proteins in fungi. Tob55 is an integral β-barrel protein with the helical N-terminal containing a polypeptide-transport-associated (POTRA) domain [16]. Tob55 belongs to the family of bacterial Omp85 that is responsible for the assembly of β-barrel proteins on bacterial OM [17]. The protein translocator of chloroplast OM, Toc75, also belongs to this group [18]. Tob55 is structurally and functionally conserved among all eukaryotes investigated so far.

Here, we identified and characterized the Tob55 homolog in *T. brucei*. TbTob55 is a MOM β-barrel protein and is crucial for survival of the procyclic form. Similar to other eukaryotes, TbTob55 is responsible for import and assembly of VDAC. In addition, TbTob55 is involved indirectly in mitochondrial presequence-containing proteins or preproteins biogenesis. These results indicate that a major protein translocator in the MOM exists in *T. brucei*.

2. Materials and methods

2.1. Strains, media, and growth

The procyclic form of *T. brucei* 427 (29-13) cell line, resistant to hygromycin and neomycin (G418), expressing the tetracycline repressor gene (TetR) and T7RNA polymerase (T7RNAP), 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) [19]. For measurement of cell growth, the procyclic cells were inoculated at a cell density of $2–3 \times 10^6$/ml in fresh medium containing appropriate antibiotics in the presence and absence of doxycycline. Cells were re-inoculated in fresh medium at each time the density reached $1–1.5 \times 10^7$/ml. Cells were harvested at different time points of growth (0–264 h) and the number of cells was counted in a Neubauer hemocytometer. To assess growth rates cumulative cell number was plotted versus time of incubation in culture.

2.2. Sequence comparison and secondary structure analysis

Amino acid sequence of TbTob55 (Tb927.3.4380) from the GeneDB database was compared for homology using BLAST analysis. Sequence comparison among Tob55s from *T. brucei*, *Saccharomyces cerevisiae*, *Neurospora crassa* and *Homo sapiens* was performed using Clustal W alignment program [20] in MacVector 10.0. The Hidden Markov Models (HMM) were built using HMMER 2.3.2 (http://hmmer.janelia.org). The prediction of secondary and tertiary structures of TbTob55 was performed using PRED-TMββ [21] and TMB-Pro prediction tools [22], respectively. The phylogenetic analysis was carried out by maximum likelihood alignments employing PhyML 3.0 [23]. WAG substitution matrix with eight rate categories with the proportion of invariable sites was estimated from the data. Tree-Puzzle 5.2 [24] was used to calculate distances between sequences and puzzleboot (shell script by A. Roger and M. Holder, http://www.tree-puzzle.de) was used to calculate bootstrap values using the same conditions. Trees were inferred using weighbor 1.2 [25]. MrBayes 3.1.2 [26] was used for performing Bayesian analysis and the Markov Chain Monte Carlo analysis was carried out for 100,000 cycles (sampling every 1000 cycles) after which the significance values were less than 0.05. The tree was drawn using Dendroscope 2.3 [27]

2.3. Generation of the inducible TbTob55RNAi and TbTob55 over-expressing cell lines

To prepare the construct for *TbTob55* double stranded RNA expression, the 554 bp fragment of the coding region of *TbTob55* was PCR amplified from the *T. brucei* genomic DNA using high fidelity Pfu polymerase (Stratagene). Sense and antisense primers containing restriction sites at 5′ ends were as follows; TbTob55 For: 5′-ATGGATCCACATTCATCCCCGTGTTATCAGTC-3′ and TbTob55 Rev: 5′-GAAAGCTTAGGCAGATTCGTTCCTTCCCTC-3′. The amplified product was cloned into the BamHI/HindIII sites of a tetracycline inducible dual promoter plasmid vector p2T7Ti-177 [28]. This construct generated *TbTob55* double stranded RNA from two opposing tetracycline regulated T7 promoter/primer and

expressed the phleomycin resistant gene constitutively for selection purposes. TbVDAC RNAi cell line was developed using the same vector as described [29]. The TbTob55 over expression construct was generated using the pLew-Myc vector [30]. The entire open reading frame of TbTob55 was PCR amplified from *T. brucei* genomic DNA using primers TbTob55-Myc For: 5′-GATCAAGCTTATGACCTTTTCTAGCGAGAGTAGTG-3′ and TbTob55-Myc Rev: 5′-CTAGTCTAGACATGGAAAAAGCGGATGACCATG-3′. The PCR product was cloned between HindIII and Xba1 site. From this construct, the C-terminal Myc-tagged Tob55 was expressed upon induction with doxycycline. The construct for *TbTob55* RNAi and TbTob55-Myc was verified by sequencing. The purified plasmid DNA was linearized by Not I. The linearized plasmid was used for transfection into procyclic cells (Tb427 29-13 expressing T7 polymerase and tetracycline repressor proteins) according to standard protocols [19].

2.4. RNA and protein analysis

RNA was isolated from the procyclic trypanosomes grown for 4 days with or without doxycycline, using Trizol reagent (Invitrogen) and Northern blot analysis was performed as described [31]. Total cellular proteins and proteins from isolated mitochondria were analyzed on SDS-PAGE (10 or 15%) as described [32], and transferred to nitrocellulose membranes at 4 °C (100 V with 25 mM Tris–HCl and 192 mM glycine, 20% v/v methanol pH 8.3) [33]. Blots were treated with polyclonal antiserum against *T. brucei* Cyt c1 [34], COIV [35], Cyt c [36], VDAC [29], Hsp70 [37], Tim17 [6], TbPP5 [38], and the *S. cerevisiae* Tob55 [39] (all at 1:1000 dilution in blocking buffer). TAO [40] was detected with the corresponding monoclonal hybridoma supernatants (1:50 dilution in 10 mM Tris–HCl, 150 mM NaCl, pH 8.0 (TBST)). Monoclonal antibody against *T. brucei* β-tubulin [41] was used in 1:20,000 dilution in TBST. Blots were treated with appropriate secondary antibody and developed using enhanced chemiluminescence (ECL) detection system (Amersham).

2.5. Isolation and post-isolation treatments of mitochondria

Mitochondria were isolated after lysis of cells via nitrogen cavitation in isotonic buffer as described [42,43]. The isolated mitochondria were stored at a protein concentration of 10 mg/ml in SME (250 mM sucrose, 20 mM MOPS/KOH, 2 mM EDTA, pH 7.4) buffer containing 50% glycerol at −70 °C. Before using, mitochondria were washed twice with 9 volumes of SME to remove glycerol. For alkali extraction, isolated mitochondria (100 μg) were treated with 100 μl of 100 mM Na_2CO_3 at pH 11.5 for 30 min on ice [42]. The supernatant and pellet fractions were collected after centrifugation (13,000 rpm for 10 min at 4 °C) and analyzed by SDS-PAGE and immunoblotting. Protease digestion of isolated mitochondria was performed by treating mitochondria (50 μg) with various concentrations of proteinase K (0–150 μg/ml) in 100 μl reaction volume of SME for 30 min on ice. After the treatment, proteinase K was inhibited by PMSF (4 mM final concentration). Mitochondria were re-isolated and the proteins were analyzed by immunoblot analysis.

2.6. In vitro protein import assay

The open reading frames (ORFs) for the COIV, TAO, and VDAC were amplified from *T. brucei* genomic DNA using the following forward and reverse primer pairs, COIVFor: 5′AGAAGCTTATGTTT-GCTCGCCGCT3′; and COIVRev: 5′AAGAATTCCTAAATCTTGTTTGA3′, TAOFor: 5′AGAAGCTTATGTTTCGTAAC3′; TAORev: 5′AAGAATTCT-TACTCGTGTTTG3′; and VDACFor: 5′GATCAAGCTTATGTTTGCACG-TGCAAAATCC3′, and VDACRev: 5′GATCGGATCCCTACGAATGGGTA-