Interactions of small Tims in *Trypanosoma brucei*

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ABSTRACT

Trypanosoma brucei causes a deadly disease known as trypanosomiasis that affects the rural population in Sub-Saharan Africa. *T. brucei* contains a single mitochondrion which needs to import thousands of proteins for its function, making mitochondria protein import essential. In eukaryotes, the mitochondria protein import machinery is comprised of three major complexes: the translocase of the outer membrane (TOM) and two translocases of the inner membrane (TIM). A group of small Tim proteins of the intermembrane space form 2-3 heterohexameric complexes and are essential for translocation of the hydrophobic inner membrane proteins through one of the TIM. Mitochondrial protein import machinery in *T. brucei* is significantly divergent. Although *T. brucei* possesses 6 homologues of the small Tims (TbTim9, TbTim10, TbTim11, TbTim12, TbTim13, and TbTim8/13), each with a characteristic secondary structure, their functions and interaction patterns are not well understood. In contrast to yeast, all the small TbTims are associated with the single TIM complex in *T. brucei* and critical for the stability of this complex. Here, we analyze the interactions of small TbTims by yeast two-hybrid (Y2-H) analysis. Results showed that all small TbTims directly interact with each other; however, stronger interactions were found between TbTim8/13 with TbTim9 and TbTim10. To determine the structural domain(s) necessary for their interaction, the small TbTims were split into their N-terminal and C-terminal helices to use for Y2-H analysis in all sorts of combinations. Results show that both helices of TbTim9, TbTim10, and TbTim8/13 are involved in interaction among themselves and with the other small TbTims, like TbTim11, TbTim12, and TbTim13, suggesting that TbTim9, TbTim10, and TbTim8/13 are the core of the small TbTim complex.

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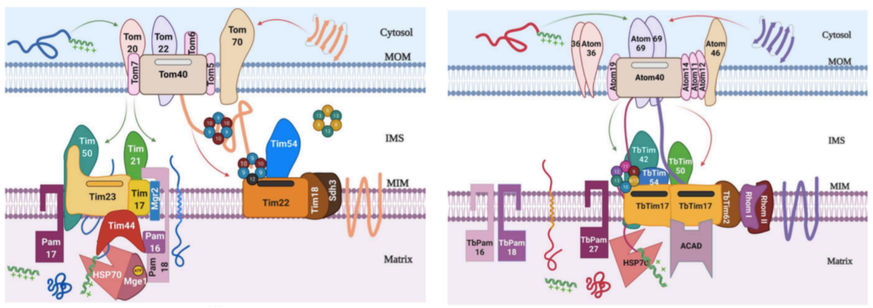
INTRODUCTION

Mitochondria perform various functions in cell; it produces energy, is involve in apoptosis, immune response and many other metabolic activities. To perform all these functions mitochondria have to import the majority of its protein from the cytosol. Thus mitochondrial protein import is essential for cell survival. *Trypanosoma brucei* (*T. brucei)* is a parasitic protozoan that causes the African sleeping sickness or African trypanosomiasis. The parasite is transmitted to the human host by the tsetse fly, which is found in Sub-Sahara Africa. *T. brucei* belongs to an eukaryotic supergroup excavate and possesses a single elongated mitochondrion. Similar to other eukaryotes *T. brucei* also import several hundred nuclear-encoded proteins into mitochondria, however, unlike to fungi and human, mitochondrial protein import machinery in *T. brucei* is quite divergent. We are studying the import machinery in the mitochondrion of *T. brucei* with an ultimate goal to understand this essential-to-life process in this parasite and also to find suitable targets for novel chemotherapeutic intervention.

Mitochondrial protein import machinery

S. cerevisiae *T. brucei*



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The small Tims are soluble proteins in the inter membrane space (IMS). They form hetero-heteromeric complexes made of three monomers of each small Tim linked alternatively, such as Tim9-Tim10 complex, Tim9-10-12 complex and Tim8-Tim13 complex. The heterohexameric complexes of the small Tims form a hydrophobic pocket that helps in the transport of polytopic inner membrane proteins across the IMS to the TIM22 complex. Each small Tim has 4 conserved cysteine residues in a pair of CX3C motifs, which are involved in formation of two intramolecular disulfide bonds. Each monomer consists of 2 α-helices connected by a loop structure. The charged residues surrounding the second CX3C motif are in involved in the formation of salt bridges. Tim9-10 complex in yeast is essential for cell survival and small molecules inhibitors have been identified.
T. brucei has 6 small TbTims: TbTim9, TbTim10, TbTim11, TbTim12, TbTim13, and TbTim8/13. All of these proteins have a pair of CX3C motifs except for TbTim12 which only possess a single C in each CX3C motif. Primary sequences are significantly divergent but the predicted secondary structures are relatively conserved. All of these are associated with TbTim17 protein complex and also to each other. Knockdown of any of these severely reduced the levels of TbTim17 and TbTim17 protein complexes. All of these are required for procyclic cell growth. These small TbTims are critical for import, assembly and stability of the TbTim17 complexes.

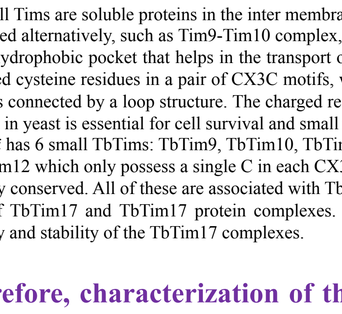
Therefore, characterization of the structure and intermolecular interaction pattern of these small TbTims are critical for not only to increase our understanding of mitochondrial protein import machinery in *T. brucei*, but also to develop novel inhibitors for this essential cellular process to combat African trypanosomiasis.

HYPOTHESIS

Small TbTims have unique structural domains necessary for their intermolecular interactions and function

METHODS

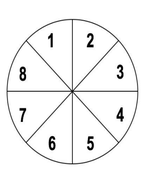
Yeast two Hybrid (Y2H) analysis



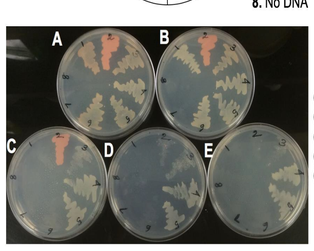
The small TbTims were cloned in AD and BD plasmids and were co-transfected in different combination in *S. cerevisiae* Y2H Gold strain. Then, the transfected yeast cells were plated on –leu/-trp/-his agarose plates to determine protein-protein interactions among the small TbTims. AT was used in increasing amounts to determine the strength of the interactions of the small TbTims. Yeast cells transfected with the AD and BD clones was plated in –leu and -trp agar plates, respectively to check the efficiency of transfection. To make sure that the small TbTim proteins are expressed in yeast, we will perform immunoblot analysis of co-transformed yeast cells using the anti-Myc and anti-HA antibodies. For negative control, the empty AD and BD plasmids was transfected into Y2H Gold cells and plated in –leu/-trp/-his plates. The cDNA for p53 and SV40 T antigen cloned in AD and BD plasmids, respectively [as obtained with the Y2-H analysis kit, Clonetech], were used as the positive controls.

RESULTS

Yeast 2-hybrid analysis of the small TbTims



1. TbTim9-AD + TbTim9-BD
2. TbTim10-AD + TbTim10-BD
3. TbTim8/13-AD + TbTim8/13-BD
4. TbTim9-AD + TbTim8/13-BD
5. TbTim10-AD + TbTim8/13-BD
6. TbTim9-AD + TbTim10-BD
7. Empty vector
8. No DNA



(A) SD –Leu/ –Trp
(B) SD –Leu/ –Trp/ –His
(C) SD –Leu/ –Trp/ –His + **2.0 mM AT**
(D) SD –Leu/ –Trp/ –His + **3.5 mM AT**
(E) SD –Leu/ –Trp/ –His + **5.0 mM AT**

Figure 1. **Yeast 2-hybrid analysis of TbTim9, TbTim10 and TbTim8/13 in different combination.** Yeast co-transformed with the small TbTim pGADT7 (activation domain, AD) and pGBKT7 (DNA-binding domain, BD) plasmids were grown in (A) synthetic-defined (SD) medium lacking leucine and tryptophan (–leu/–trp), (B) medium lacking leucine, tryptophan, and histidine (–leu/–trp/–his), (C) –leu/–trp/–his medium containing 2.0 mM 3-amino-1,2,4-triazole (AT), (D) –leu/–trp/–his medium containing 3.5 mM AT, and (E) –leu/–trp/–his medium containing 5.0 mM AT. The schematic of small TbTim AD and BD plasmid combinations for co-transformation are shown. Yeast cells co-transformed with empty vector (pGADT7) or and no DNA were used as negative controls. Yeast cells co-transformed with SV40-AD and p53-BD was used as positive control. The plates shown here were obtained from one of three independent experiments.

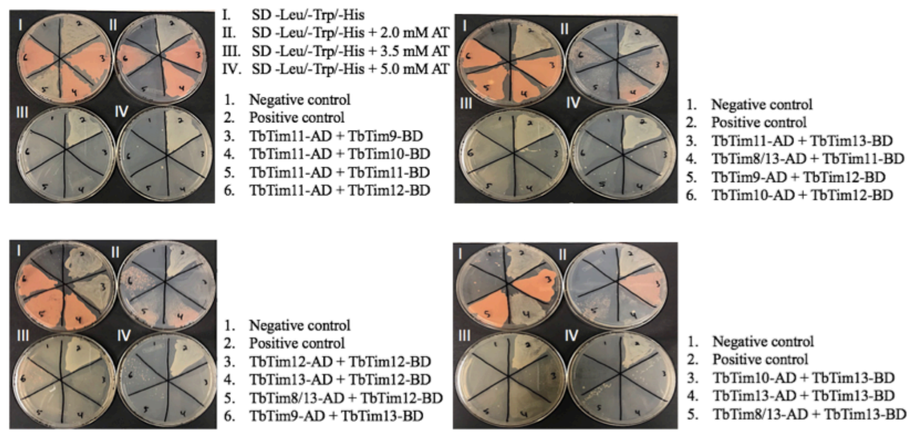


Table 1: Yeast 2-hybrid data analysis of all the possible combination of the small TbTims

Interacting pairs	Growth				Interacting pairs	Growth			
	0 mM AT	2.0 mM AT	3.5 mM AT	5.0 mM AT		0 mM AT	2.0 mM AT	3.5 mM AT	5.0 mM AT
TbTim9-AD + TbTim9-BD	+	-	-	-	TbTim10-AD + TbTim11-BD	+	+/-	-	-
TbTim10-AD + TbTim10-BD	+	+	-	-	TbTim12-AD + TbTim10-BD	+	+/-	-	-
TbTim8/13-AD + TbTim8/13-BD	+	+	+	+/-	TbTim13-AD + TbTim10-BD	+	+/-	-	-
TbTim11-AD + TbTim11-BD	+	-	-	-	TbTim10-AD + TbTim8/13-BD	+	+	+	+
TbTim12-AD + TbTim12-BD	+	-	-	-	TbTim12-AD + TbTim11-BD	+	+/-	-	-
TbTim13-AD + TbTim13-BD	+	+	+	-	TbTim13-AD + TbTim11-BD	+	+/-	-	-
TbTim9-AD + TbTim10-BD	+	-	-	-	TbTim11-AD + TbTim8/13-BD	+	+/-	-	-
TbTim9-AD-TbTim11-BD	+	+/-	-	-	TbTim12-AD + TbTim13-BD	+	+	-	-
TbTim12-AD + TbTim9-BD	+	+/-	-	-	TbTim12-AD + TbTim8/13-BD	+	+/-	-	-
TbTim13-AD + TbTim9-BD	+	-	-	-	TbTim13-AD + TbTim8/13-BD	+	+/-	-	-
TbTim9-AD + TbTim8/13-BD	+	+	+	+					

Table 2. Summary result of TbTim8/13 split helices Y2H analysis

Interacting pairs	Growth				Interacting pairs	Growth			
	0 mM AT	2.0 mM AT	3.5 mM AT	5.0 mM AT		0 mM AT	2.0 mM AT	3.5 mM AT	5.0 mM AT
TbTim8/13H1-AD + TbTim9H1-BD	+	+	+	+/-	TbTim8/13H2-AD + TbTim9H1-BD	+	-	-	-
TbTim8/13H1-AD + TbTim9H2-BD	+	-	-	-	TbTim8/13H2-AD + TbTim9H2-BD	+	-	-	-
TbTim8/13H1-AD + TbTim10H1-BD	+	+/-	-	-	TbTim8/13H2-AD + TbTim10H1-BD	+	+	+	+/-
TbTim8/13H1-AD + TbTim10H2-BD	+	+/-	-	-	TbTim8/13H2-AD+ TbTim10H2-BD	+	-	-	-
TbTim8/13H1-AD + TbTim11H1-BD	+	+	+	+/-	TbTim8/13H2-AD + TbTim11H1-BD	+	+/-	-	-
TbTim8/13H1-AD + TbTim11H2-BD	+	+	+/-	-	TbTim8/13H2-AD + TbTim11H2-BD	+	+/-	-	-
TbTim8/13H1-AD + TbTim12H1-BD	+	+	+	+	TbTim8/13H2-AD + TbTim12H1-BD	+	-	-	-
TbTim8/13H1-AD + TbTim12H2-BD	+	+	+	+	TbTim8/13H2-AD + TbTim12H2-BD	+	-	-	-
TbTim8/13H1-AD + TbTim13H1-BD	+	+/-	-	-	TbTim8/13H2-AD + TbTim13H1-BD	+	+/-	-	-
TbTim8/13H1-AD + TbTim13H2-BD	+	+/-	-	-	TbTim8/13H2-AD + TbTim13H2-BD	+	+/-	-	-
TbTim8/13H1-AD + TbTim8/13H2-BD	+	+/-	-	-	TbTim8/13H2-AD + TbTim8/13-H1 BD	+	+/-	-	-

Table 3. Summary result of TbTim9 split helices Y2H analysis

Interacting pairs	Growth				Interacting pairs	Growth			
	0 mM AT	2.0 mM AT	3.5 mM AT	5.0 mM AT		0 mM AT	2.0 mM AT	3.5 mM AT	5.0 mM AT
TbTim9H1-AD + TbTim9H2-BD	+	-	-	-	TbTim9H2-AD + TbTim9H1-BD	+	-	-	-
TbTim9H1-AD+ TbTim10H1-BD	+	-	-	-	TbTim9H2-AD + TbTim10H1-BD	+	-	-	-
TbTim9H1-AD + TbTim10H2-BD	+	+	+	+/-	TbTim9H2-AD + TbTim10H2-BD	+	-	-	-
TbTim9H1-AD+ TbTim11H1-BD	+	+	+/-	-	TbTim9H2-AD + TbTim11H1-BD	+	-	-	-
TbTim9H1-AD + TbTim11H2-BD	+	+	-	-	TbTim9H2-AD + TbTim11H2-BD	+	+	+/-	-
TbTim9H1-AD + TbTim12H1-BD	+	+	+	+	TbTim9H2-AD + TbTim12H1-BD	+	-	-	-
TbTim9H1-AD + TbTim12H2-BD	+	+	-	-	TbTim9H2-AD + TbTim12H2-BD	+	-	-	-
TbTim9H1-AD + TbTim13H1-BD	+	+	-	-	TbTim9H2-AD + TbTim13H1-BD	+	+	+	+
TbTim9H1-AD + TbTim13H2-BD	+	-	-	-	TbTim9H2-AD + TbTim13H2-BD	+	-	-	-
TbTim9H1-AD+ TbTim8/13H1-BD	+	+	+	+/-	TbTim9H2-AD + TbTim8/13H1-BD	+	-	-	-
TbTim9H1-AD + TbTim8/13H2-BD	+	-	-	-	TbTim9H2-AD+ TbTim8/13H2-BD	+	-	-	-

Table 4. Summary result of TbTim10 split helices Y2H analysis

Interacting pairs	Growth				Interacting pairs	Growth			
	0 mM AT	2.0 mM AT	3.5 mM AT	5.0 mM AT		0 mM AT	2.0 mM AT	3.5 mM AT	5.0 mM AT
TbTim10H1-AD + TbTim9H1-BD	+	-	-	-	TbTim10H2-AD + TbTim9H1-BD	+	+	+	+/-
TbTim10H1-AD + TbTim9H2-BD	+	-	-	-	TbTim10H2-AD + TbTim9H2-BD	+	-	-	-
TbTim10H1-AD + TbTim10H2-BD	+	+/-	-	-	TbTim10H2-AD + TbTim10H1-BD	+	+/-	-	-
TbTim10H1-AD + TbTim11H1-BD	+	+	+	+	TbTim10H2-AD + TbTim11H1-BD	+	+/-	-	-
TbTim10H1-AD + TbTim11H2-BD	+	+/-	-	-	TbTim10H2-AD + TbTim11H2-BD	+	-	-	-
TbTim10H1-AD+ TbTim12H1-BD	+	+	+	+	TbTim10H2-AD + TbTim12H1-BD	+	+/-	-	-
TbTim10H1-AD + TbTim12H2-BD	+	+/-	-	-	TbTim10H2-AD + TbTim12H2-BD	+	-	-	-
TbTim10H1-AD + TbTim13H1-BD	+	+	-	-	TbTim10H2-AD + TbTim13H1-BD	+	+	-	-
TbTim10H1-AD + TbTim13H2-BD	+	-	-	-	TbTim10H2-AD + TbTim13H2-BD	+	-	-	-
TbTim10H1-AD + TbTim8/13H1-BD	+	+/-	-	-	TbTim10H2-AD + TbTim8/13H1-BD	+	+/-	-	-
TbTim10H1-AD + TbTim8/13H2-BD	+	+	+	+/-	TbTim10H2-AD+ TbTim8/13H2-BD	+	-	-	-

CONCLUSION

Yeast 2-hybrid analysis shows unique interactions between the small TbTims

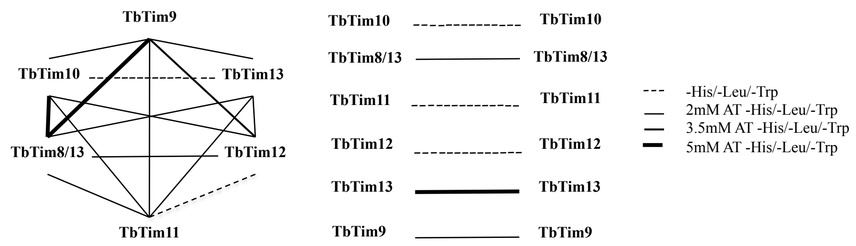


Figure 2. A schematic of small TbTim interaction pattern based on the Y2H analysis results. Solid bold lines represent stronger interaction, solid lines represent interaction and dotted lines represent weaker interaction.

Yeast two hybrid analysis shows unique interaction between the small TbTims.
- **All small TbTims could directly interact with each other and themselves**
- **Stronger interactions are found between TbTim8/13 with TbTim9 and TbTim10, as well as TbTim8/13 with itself.**

Y2H analysis shows multiple interactions of TbTim9, TbTim8/13, and TbTim10 helices with other small TbTims
- **TbTim9 helix-1 with TbTim10 helix-2 and TbTim12 helix-1**
- **TbTim9 helix-2 with TbTim13 helix-1**
- **TbTim10 helix-1 with TbTim8/13 helix-2, TbTim11 helix-1 and TbTim12 helix-1**
- **TbTim13 helix-1 with TbTim9 helix-2 and TbTim12 helix-2**
- **TbTim8/13 helix-1 with TbTim9 helix-1and TbTim12 helix-1 and helix-2**

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