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Evidence for a degradosome-like complex in the mitochondria of *Trypanosoma brucei*

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Abstract

Mitochondrial RNA turnover in yeast involves the degradosome, composed of DSS-1 exoribonuclease and SUV3 RNA helicase. Here, we describe a degradosome-like complex, containing SUV3 and DSS-1 homologues, in the early branching protozoan, *Trypanosoma brucei*. TbSUV3 is mitochondrially localized and co-sediments with TbDSS-1 on glycerol gradients. Co-immunoprecipitation demonstrates that TbSUV3 and TbDSS-1 associate in a stable complex, which differs from the yeast degradosome in that it is not stably associated with mitochondrial ribosomes. This is the first report of a mitochondrial degradosome-like complex outside of yeast. Our data indicate an early evolutionary origin for the mitochondrial SUV3/DSS-1 containing complex.

Keywords

RNA turnover; RNA processing; trypanosome; RNA helicase; exoribonuclease

1. Introduction

The degradation of RNA is an essential element in the regulation of gene expression. It both controls the abundance of mature RNAs and eliminates processing by-products and aberrant or defective molecules that form during RNA synthesis and maturation [1]. RNA degradation is of particular importance in mitochondria where transcriptional control is minimal and polycistronic transcription produces precursor RNAs that require extensive processing [2-5]. The machineries that catalyze RNA turnover in mitochondria exhibit substantial divergence between species [6]. In yeast, the mitochondrial degradosome is comprised of an RNR (RNase II/RNase R-like) family hydrolytic exoribonuclease, encoded by *DSS-1* gene, and an NTP-dependent RNA helicase, encoded by the *SUV3* gene. DSS-1 and SUV3 appear to be the sole components of the degradosome based on TAP-tagging studies in yeast, which also showed that the complex is exclusively associated with mitochondrial ribosomes [7]. The degradosome, which exhibits hydrolytic 3′ to 5′ exoribonuclease and RNA helicase activities, is the only

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MINT-7187980: *SUV3* (genbank_protein_gi:XP_844349) and *DSS1* (uniprotkb:Q38EM3) *colocalize* (MI:0403) by *cosedimentation* (MI:0027)
MINT-7188074: *SUV3* (genbank_protein_gi:XP_844349) *physically interacts* (MI:0914) with *DSS1* (uniprotkb:Q38EM3) by *anti tag coimmunoprecipitation* (MI:0007)

known exoribonuclease involved in yeast mitochondrial RNA (mtRNA) turnover [8]. *S. cerevisiae* strains that are genetically inactivated for either DSS-1 or SUV3 have similar phenotypes, strongly accumulating excised introns as well as mRNA and rRNA precursors with abnormal 5′ and 3′ termini [9-11]. These cells also display decreased steady-state levels of mature transcripts along with disruption of translation [7,11,12]. Orthologues of the SUV3 helicase are present in the genomes of a wide spectrum of eukaryotes, and they have been shown to be at least partially mitochondrially localized in humans and plants [13-15]. In contrast to yeast, however, human and plant mitochondria lack the DSS-1 exoribonuclease. They do contain the phosphorolytic exoribonuclease polynucleotide phosphorylase (PNPase), although there is no evidence for its association with SUV3 [16,17]. A recent study demonstrated that human cells depleted of the SUV3 helicase accumulate shortened poly(A+) mtRNAs and are impaired in translation [18]. These studies indicate that SUV3 can profoundly affect mitochondrial RNA metabolism in the absence of a yeast-like degradosome complex.

Trypanosoma brucei is a protozoan parasite that has consistently been identified as one of the earliest branching mitochondria-containing eukaryotes [19]. Mitochondrial RNA metabolism in *T. brucei* is extraordinarily complicated, involving polycistronic transcription, extensively overlapping genes, and massive remodeling of mRNAs by guide RNA-directed uridine insertion/deletion editing [20]. We previously identified a gene encoding a homologue of DSS-1 in the *T. brucei* genome (termed *TbDSS-1*) [21], and a peptide originating from TbDSS-1 was recently detected in the mitochondrial proteome [22]. Targeted depletion of TbDSS-1 in insect stage *T. brucei* results in aberrant levels of several mitochondrial RNA species, including never edited, unedited and edited mRNAs as well as guide RNAs [21]. TbDSS-1 depleted cells also accumulate RNA maturation by-products originating from the region upstream of the first genes on the major and minor strands of the mitochondrial genome, and 12S rRNA processing intermediates with mature 3′ ends and unprocessed 5′ ends [23]. Overall, these studies suggest that TbDSS-1 represents at least one of the main exoribonucleases involved in RNA turnover and surveillance in *T. brucei* mitochondria. In the present study, we report a *T. brucei* homologue of the SUV3 RNA helicase (TbSUV3). To determine whether TbSUV3 interacts with TbDSS-1 in a mitochondrial degradosome-like complex, we created a *T. brucei* cell line expressing a PTP (ProtC-TEV-ProtA [24]) tagged TbSUV3 protein at an endogenous allele. We show that the TbSUV3-PTP fusion protein is properly expressed and targeted to the mitochondrion. Glycerol gradient fractionation suggests that TbSUV3 and TbDSS-1 co-sediment in a high-molecular-weight complex, and subsequent IgG purification of TbSUV3-PTP containing complexes shows that the two proteins interact in *T. brucei* mitochondria. These studies represent the first report of a core enzymatic complex that is likely involved in RNA turnover and surveillance in the mitochondria of *T. brucei*. Further, this is the first report of a mitochondrial degradosome-like complex in an organism other than yeast. Our data demonstrate an early evolutionary origin for the mitochondrial SUV3/DSS-1 containing complex.

2. Materials and methods

2.1. Oligonucleotides used for 5′ RACE analysis

The oligonucleotides used for 5′ RACE are listed as follows with restriction sites underlined. CSL-22 (5′-GCATCGATGCTATTATTAGAACAGTTTCTGTACTATATTG -3′), SUV3-8 (5′-GCGGATCCAACGCCGCGTGAGTCTTCC-3′), SUV3-9 (5′-GCGGATCCGTGCCTTCGGGTACCAGTC-3′).

2.2. Trypanosome cell culture, transfection, and cell fractionation

The procyclic form (PF) *T. brucei brucei* clone IsTAR1 stock EATRO 164 was grown as previously described [25]. Stable cell lines constitutively expressing a TbSUV3 C-terminal

PTP tag fusion protein were generated via electroporation. To generate the pC-PTP-TbSUV3 construct, a 500-nucleotide fragment of TbSUV3 C-terminal coding region was PCR amplified using TbSUV3-PTP5′ (5′-GCCGGGGCCCAAGACCTCAGGTGTGGTGCC-3′) forward and TbSUV3-PTP3′ (ATAAGAATGCGGCCGCGGCAACCTCCGCAACAGCTC-3′) reverse primers and cloned into the ApaI /Not l restriction sites of the pC-PTP-Neo vector [24] (a generous gift from Arthur Günzl, Univ. of Connecticut). For genomic integration, pTbSUV3-PTP-NEO was linearized within the TbSUV3 sequence at a unique Bcl I restriction site. For transfection, log-phase PF *T. brucei* clone IsTAR1 stock EATRO 164 cells were electroporated in the presence of twenty micrograms of Bcl I linearized TbSUV3-PTP. Transfections were carried out on ice in 2-mm cuvettes using a Bio-Rad electroporator with two pulses at the following settings: 800 V, 25 μF, and 400 Ω. Following transfection, cells were selected with 40 μg of G418/ml and clonal cell lines were generated by limiting dilution. Expression of PTP-tagged protein was analyzed by western blotting with PAP probe (Sigma), which detects the Protein A domain of the PTP tag.

Mitochondria were isolated by the procedure of Harris, *et al.* [26]. Whole cell and cytoplasmic fractionation was carried out using the procedure of Zeiner, *et al.* [27]. The degree of cytoplasmic contamination of the mitochondrial preparation was assessed by western blotting using antibodies against cytoplasmic TbHsp70.4.

2.3. Glycerol gradient sedimentation

Glycerol gradient fractionation of mitochondrial lysates was performed as previously described [28]. Mitochondrial lysate was obtained from 10^{10} PF *T. brucei* cell equivalents by adding 500 μl of mitochondrial lysis buffer (20mM Tris-HCl (pH 7.5), 50 mM KCl, 10mM $MgCl_2$, 100 μM ATP, 0.2% NP-40, Complete EDTA-free protease inhibitors (Roche)) to purified mitochondria and incubating on ice for 10 min prior to centrifugation at 13,000 × g for 15 min. Five hundred μl of purified mitochondrial lysate was layered onto a 12-ml 5-20% linear glycerol gradient and centrifuged for 20 h at 4°C in Beckman SW-41 rotor at 35,000 rpm. Twenty-four 500 μl fractions were collected from the top of the tube, and 20 μl of each fraction was analyzed by western blotting with PAP reagent for the detection of TbSUV3 and polyclonal anti-TbDSS-1 antibodies [21] to detect endogenous TbDSS-1. Standards (cytochrome c, 1.9S; bovine serum albumin, 4S; yeast alcohol dehydrogenase, 7.4S; catalase, 11S; and thyroglobulin, 19S) were fractionated in a parallel gradient and analyzed by SDS-PAGE and Coomassie blue staining.

2.4. Immunoprecipitation of the TbSUV3 containing complex

For IgG purification of TbSUV3-PTP, peak glycerol gradient fractions (7-13) were pooled and fresh protease inhibitor tablet was added. Five hundred μl of pooled gradient fractions was incubated with 20 μl IgG Fastflow Sepharose beads (Amersham Biosciences) for 2 h at 4°C. The bound material was pelleted by centrifugation at 10,000 × g for 5 min and the unbound supernatant transferred to a separate tube. The Sepharose beads were then washed three times with PA-150 buffer (20mM Tris-HCl (pH 7.7), 150 mM KCl, 3 mM $MgCl_2$, 0.5 mM DTT, 0.1% Tween20) and resuspended in Buffer A (10mM Tris-HCl (pH 8.0), 25mM KCl, 10mM $MgCl_2$). The IgG Sepharose beads were boiled at 95°C for 5 min prior to loading on SDS-PAGE. Ten percent of each fraction was analyzed by western blot to detect TbDSS-1 and TbSUV3-PTP using anti-TbDSS1 antibodies [21] and PAP reagent, respectively.

Immunoprecipitations using anti-ProtC antibodies were performed as described above with the following modifications. Ten μg of anti-ProtC antibody (Roche monoclonal HPC4) was incubated with 500 μl of pooled gradient fractions in 2 mM $CaCl_2$ with rocking at 4°C for 2 h. Control reactions in the absence of antibody were processed in parallel. Twenty μl of Protein G Sepharose beads were then added to each tube and the slurry was incubated with rocking

for an additional 2 h at 4°C. The bound material was pelleted by centrifugation at 10,000 × g for 5 min and unbound supernatant transferred to a separate tube. The Sepharose beads were then washed three times with PC-150 buffer (20mM Tris-HCl (pH 7.7), 150 mM KCl, 3 mM $MgCl_2$, 1mM $CaCl_2$, 0.1% Tween20), resuspended in buffer A and boiled at 95°C for 5 min prior to loading on SDS-PAGE. Ten percent of each fraction was analyzed by western blot as described above.

3. Results and discussion

3.1 TbSUV3 encodes a putative ATP-dependent RNA helicase that localizes to the mitochondria

We previously described the role of the mitochondrial exoribonuclease, TbDSS-1, in RNA stability and as part of an RNA surveillance system that eliminates stalled 12S rRNA processing intermediates and maturation by-products from the system [21,23]. To determine if *T. brucei* possesses an SUV3 homologue that might act in concert with TbDSS-1, analogous to the yeast mitochondrial degradosome, we searched *T. brucei* GeneDB with the *S. cerevisiae* SUV3 sequence. We identified a predicted protein highly homologous to SUV3, which we term TbSUV3 (Tb927.4.1990). To confirm its expression and characterize the corresponding mRNA, we performed 5′ RACE on oligo(dT)-primed PF RNA using a gene-specific primer in combination with a primer homologous to the 39 nt spliced leader sequence present on all *T. brucei* nuclear encoded mRNAs. This analysis indicated the presence of a mature *trans* spliced TbSUV3 mRNA with an 18 nt 5′ untranslated region. To confirm the open reading frame (ORF) identified *in silico*, the predicted ORF was PCR amplified from PF *T. brucei* cDNA, cloned, and sequenced. The 1,881-nt ORF is identical to the sequence in *T. brucei* Gene DB with the exception of an alanine to valine substitution at amino acid 617, and predicts a protein with a molecular mass of 70.6 kDa and a pI of 9.01. The predicted protein exhibits 32-38% identity and 50-54% similarity with SUV3 homologues from *Homo sapiens, Saccharomyces cerevisiae Arabidopsis thaliana*, and *Caenorhabditis elegans* [15] over the conserved central region (Fig. 1A). SUV3-like proteins form a distinct and conserved Ski2p family of DExH/D RNA helicases that possess seven conserved motifs within their central region. Depicted in Fig. 1B is a sequence alignment of Motifs I to VI of SUV3 proteins from *T. brucei*, *H. sapiens*, *S. cerevisiae*, *A. thaliana*, and *C. elegans*. Motifs I and II, also known as the Walker A and B box, are crucial for ATPase and helicases activities [29]. Motifs Ia and Ib are thought to be involved in RNA binding in association with motifs IV and V, although this has only been demonstrated for eIF4A [30]. Interestingly, Motif III is characteristically absent in all SUV3 helicases, including TbSUV3. Together, these analyses indicate that *T. brucei* expresses an SUV3 homologue.

We next wanted to confirm the expected mitochondrial localization of TbSUV3. TbSUV3 is predicted to contain a mitochondrial import sequence at its N-terminus by the PSORTII program (http://psort.ims.u-tokyo.ac.jp/). In addition, the N-terminus of the TbSUV3 ORF exhibits characteristic regions of homology to known trypanosome mitochondrial import sequences [21,31,32], including tandem arginine residues followed by multiple hydrophobic amino acids with interspersed and flanking serine and threonine residues (Fig. 2A). To determine whether TbSUV3 is mitochondrially localized, we used epitope tagging to create a TbSUV3 fusion protein in *T. brucei*. We utilized a construct designed for stable integration of a PTP tag into an endogenous allele [24], which allows PTP-tagged proteins to be expressed at normal levels, potentially alleviating problem of nonspecific protein-protein interactions due to overexpression of the tagged protein. To this end, a 500-bp fragment of the TbSUV3 C-terminal coding region was cloned into pC-PTP-Neo vector [24]. The resulting vector was linearized within the TbSUV3 sequence at a unique restriction site and transfected into *T. brucei* EATRO 164 strain. Cultures that had integrated the plasmid construct into an endogenous TbSUV3 allele were selected by G418 treatment, and clonal lines subsequently

isolated by limiting dilution. Western blot analysis using the PAP reagent, which recognizes the Protein A domain in the PTP tag, confirmed that a protein of the correct size (~84 kDa) was expressed in TbSUV3-PTP cells (Fig. 2B, top panel, compare lanes 1 and 2). Total protein, cytosolic, and mitochondrial extracts were prepared from TbSUV3-PTP cells, and equal microgram amounts of protein were analyzed using the PAP reagent. TbSUV3-PTP was detected only in the total protein and mitochondrial fractions (Fig. 2B, lanes 2 and 4), and not in the cytosolic fraction (Fig. 2B, lane 3). Moreover, TbSUV3-PTP was enriched in mitochondria compared to whole cells, demonstrating that it is targeted to the mitochondrion. Western blotting using anti-TbHsp70.4 (Fig. 2B, bottom panel) confirmed the efficiency of the mitochondrial fractionation. The presence of a putative mitochondrial import sequence and enrichment in mitochondrial extracts, lead us to conclude that TbSUV3 is a nuclear encoded, mitochondrially localized protein. Consistent with this conclusion, a recent survey of the *T. brucei* mitochondrial proteome identified several TbSUV3 peptides [22].

3.2 TbSUV3 is present in higher order complexes

To address whether TbSUV3 is present in a macromolecular complex, we subjected mitochondrial extract from cells expressing TbSUV3-PTP to glycerol gradient fractionation. Fractions from a 5 to 20% glycerol gradient were first resolved by SDS-PAGE and analyzed by western blot with the PAP reagent (Fig. 3, top panel). TbSUV3-PTP exhibited a broad distribution in fractions 5 to 19, sedimenting predominantly between 8 and 11.3S. Monomeric TbSUV3-PTP was expected to exhibit sedimentation values of less than 7.4S, suggesting the presence of TbSUV3-containing macromolecular complexes.

Previous glycerol gradient analysis demonstrated that TbDSS-1 is also present in higher order complexes, but unlike yeast DSS-1, is not stably associated with ribosomes [21]. To determine whether the sedimentation profile of TbDSS-1 overlaps or coincides with that of TbSUV3-PTP, we analyzed the same gradient fractions with antibodies against TbDSS-1. HRP conjugated goat anti-rabbit antibodies, which would not be expected to interact with the Protein A portion of the TbSUV3-PTP protein [33], were utilized as secondary antibodies. As shown in Fig. 3 (bottom panel), TbDSS-1 exhibits a broad sedimentation profile in fractions 7-17, corresponding to 8 to 10S, similar to TbSUV3-PTP. These results suggest that TbSUV3 and TbDSS-1 may be associated in mitochondrial complexes.

3.3 TbSUV3 interacts with TbDSS-1 in a mitochondrial degradosome-like complex

To determine whether TbSUV3 and TbDSS-1 are stably associated, we performed co-immunoprecipitation experiments. Peak glycerol gradient fractions (fractions 7-13, Fig. 3) were pooled and incubated with IgG Sepharose beads, which bind the Protein A moiety of the PTP tag. After washing, ten percent of the input, unbound, and bound proteins were analyzed by western blot with the PAP reagent to confirm TbSUV3-PTP precipitation and with anti-TbDSS-1 antibodies to assess their interaction. As shown in Fig. 4A, the majority of both TbSUV3 and TbDSS-1 were identified in the pellet, demonstrating an *in vivo* association of the two proteins. To confirm this interaction, we performed co-immunoprecipitation experiments with the same pooled gradient fractions and anti-ProtC antibodies directed against the ProtC moiety of the PTP tag. TbSUV3-PTP was precipitated using the anti-ProtC antibodies bound to Protein G Sepharose beads, and negative control experiments were performed in the absence of anti-ProtC antibodies to account for non-specific binding of TbDSS-1 to the beads. In these experiments, we detected both TbSUV3-PTP and TbDSS-1 in the pellet in the presence of anti-ProtC antibody, but not in controls performed in the absence of primary antibody (Fig. 4B). Collectively, these results demonstrate that TbSUV3 associates with TbDSS-1 in *T. brucei* mitochondria.

3.4. Concluding remarks

This study reports the expression of an SUV3 RNA helicase homologue, TbSUV3, in the early branching eukaryote, *T. brucei*. We further demonstrate that TbSUV3 is localized to mitochondria where it associates with the TbDSS-1 exoribonuclease in a complex analogous to the *S. cerevisiae* mitochondrial degradosome [7]. This constitutes the first report of a DSS-1/SUV3 containing complex in an organism other than yeast. While we were unable to isolate enzymatically active TbSUV3 or TbDSS-1 from trypanosomes or bacteria, presumably due to the lability of these proteins, both proteins possess all of the residues necessary for their predicted enzymatic functions, indicative of a role in mitochondrial RNA metabolism. We were also unable to obtain TbSUV3 depleted cells for functional studies despite exhaustive attempts. Nevertheless, TbDSS-1 knockdown cells display pleitropic effects on trypanosome mitochondrial RNA metabolism similar to those described in DSS-1 or SUV3 null yeast cells [7,21,23]. Thus, our data reveal an early evolutionary origin for the mitochondrial degradosome. This complex has apparently been lost during evolution, as human SUV3 functions in mitochondrial RNA metabolism in the absence of any DSS-1 homologue [18]. It will be of future interest to determine the spectrum of organisms that possess a mitochondrial DSS-1/SUV3 containing complex, and to better understand the myriad functions of this important complex *in vivo*.

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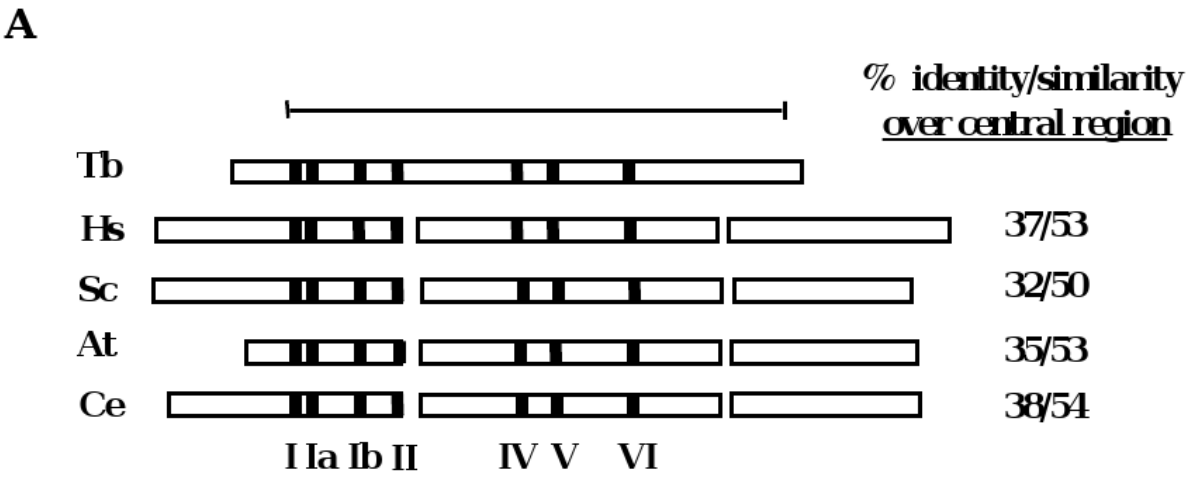

B

Motif I (Walker A Box, ATP binding)

Tb	134	GPTNSGKT	141
Hs	207	GPTNSGKT	214
Sc	239	GPTNSGKT	246
At	96	GPTNSGKT	103
Ce	196	GPTNSGKT	203

Motifs Ia and Ib (RNA binding)

Tb	156	YCAPIKAL	163	197	TVE	199
Hs	229	YCGPIKLL	236	275	TVE	277
Sc	261	YAGPIRLL	268	308	TVE	310
At	118	YCGPIRLL	125	160	TVE	162
Ce	218	FCGPIKLL	225	264	TVE	266

Motif II (Walker B Box, NTP binding/hydrolysis)

Tb	213	DEVQ	216
Hs	291	DEIQ	294
Sc	323	DEIQ	326
At	176	DEIQ	179
Ce	180	DEIQ	183

Motif IV

Tb	336	FNRG	339
Hs	411	FNDP	414
Sc	447	FNNG	450
At	297	FNDE	300
Ce	400	FNDP	403

Motif V (RNA/NTP binding)

Tb	363	GLNM	366
Hs	430	GLNL	433
Sc	463	GLNL	466
At	316	GLNL	319
Ce	419	GLNL	422

Motif VI (RNA binding)

Tb	394	QVAGRAGR	401
Hs	466	QIAGRAGR	473
Sc	494	QIGGRAGR	501
At	347	QIAGRAGR	354
Ce	446	QIAGRAGR	453

Fig. 1.

TbSUV3 is a putative ATP-dependent RNA helicase with conserved motifs. (A) Schematic representation of the structure of SUV3 homologues from *T. brucei* (Tb; accession no. XP844349), *H. sapiens* (Hs; accession no. NP_003162), *S. cerevisiae* (Sc; accession no. NP015296), *A. thaliana* (At; accession no. BAF01552), and *C. elegans* (Ce; accessin no. NP001040911) illustrating the presence of conserved motifs (Motifs I, Ia, Ib, II, IV, V, and VI) and variable N- and C-termini. The percent amino acid identity/similarity over the conserved central region (encompassing amino acids 115 to 602 of TbSUV3) is shown. (B) A multiple sequence alignment of Motifs I-VI of SUV3 homologues shown in panel A was

generated using ClustalW. Identical amino acids are indicated by white letters on a black background; similar amino acids are indicated by white letters on a gray background.

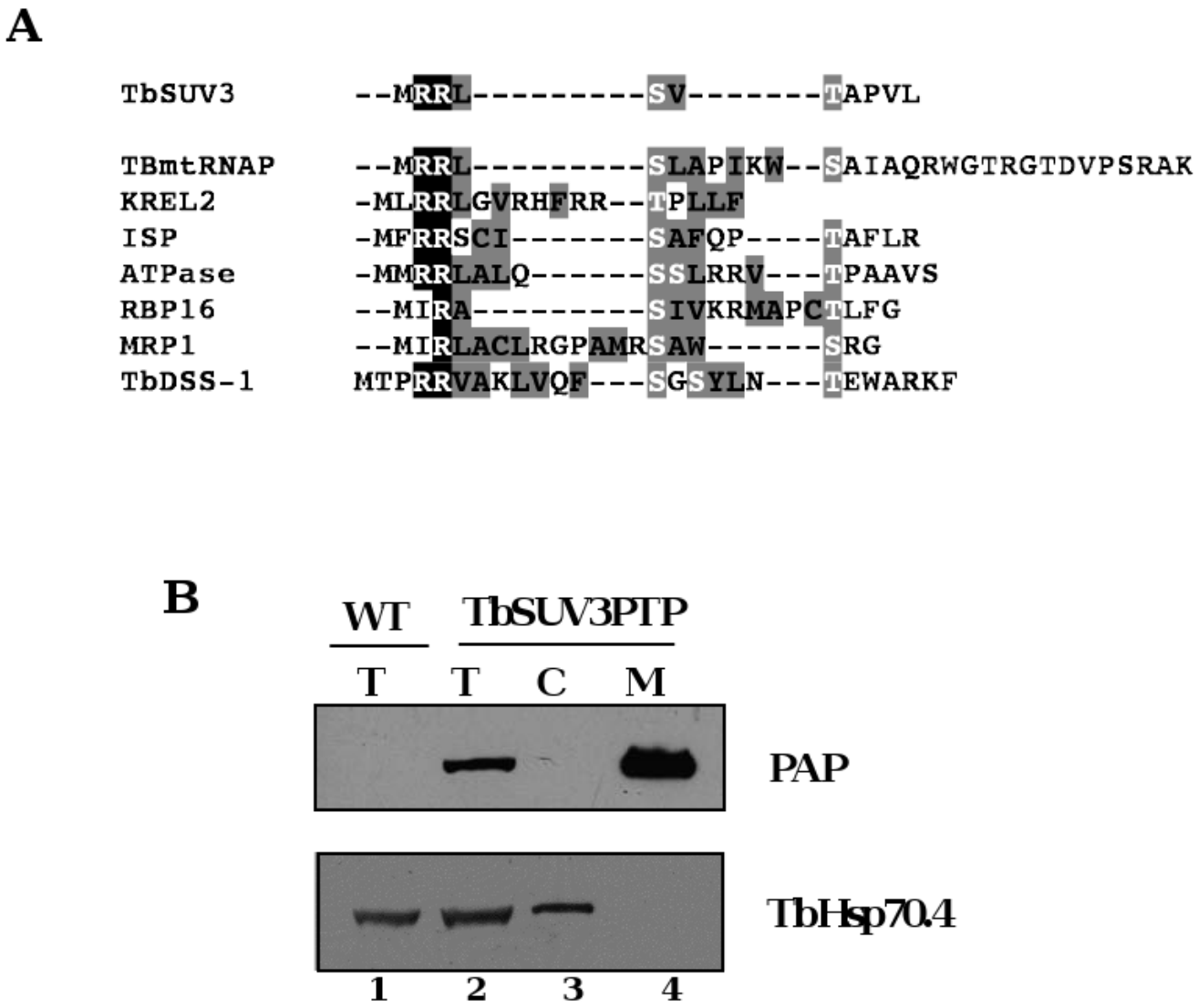

Fig. 2.

TbSUV3 is mitochondrially localized. (A) Sequence alignment of the TbSUV3 N terminal amino acid sequence with known and predicted targeting sequences from *T. brucei* mitochondrial proteins TBmtRNAP, KREL2, ISP, ATPase subunit 9, RBP16, MRP1, and TbDSS-1. The characteristic one or two N-terminal arginine residues are shown by white letters on black background. Serine and threonine residues (white letters on gray background) flank hydrophobic residues that are shown by black letters on gray background. (B) Mitochondrial localization of TbSUV3-PTP fusion protein. Wild type (WT) or TbSUV3-PTP expressing cells were analyzed by western blot with the PAP reagent to detect TbSUV3-PTP (top) or with antibody against TbHSP70.4 as a cytoplasmic marker (bottom). Each lane contains 10 μg protein. Total cell extracts (T, lanes 1 and 2); cytoplasmic extract (C, lane 3); mitochondrial extract (M, lane 4).

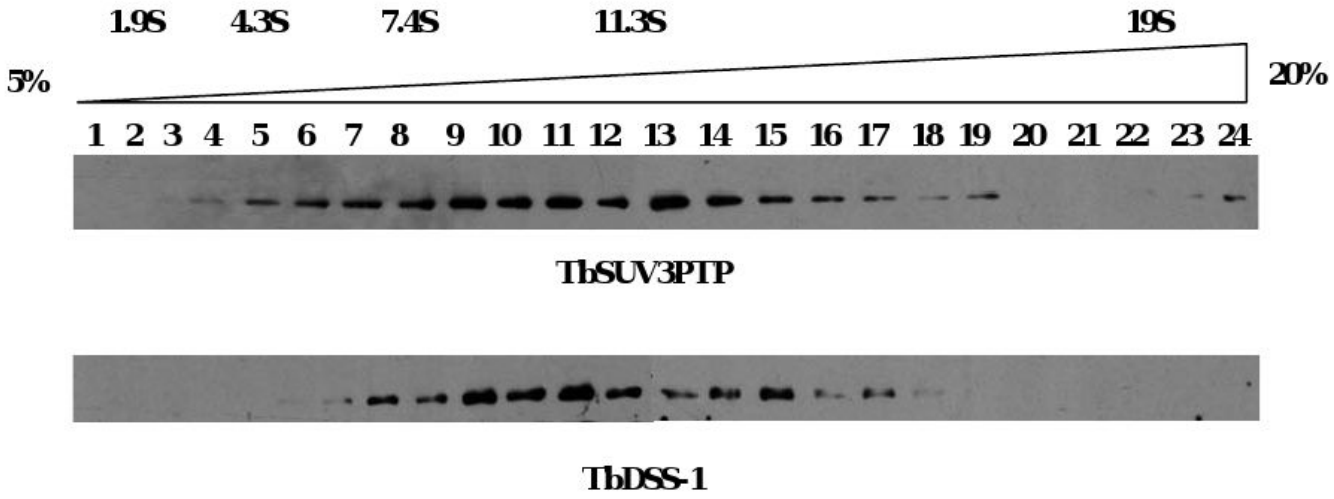

Fig. 3.

TbSUV3 and TbDSS-1 co-sediment in glycerol gradients. Mitochondrial extracts from TbSUV3-PTP expressing cells were fractionated on a 5 to 20% glycerol gradient. Aliquots of each fraction were analyzed by western blot using the PAP reagent to detect TbSUV3-PTP (top panel) and antibodies against TbDSS-1 (bottom panel). Sedimentation coefficients were determined by sedimentation of markers in a parallel gradient.

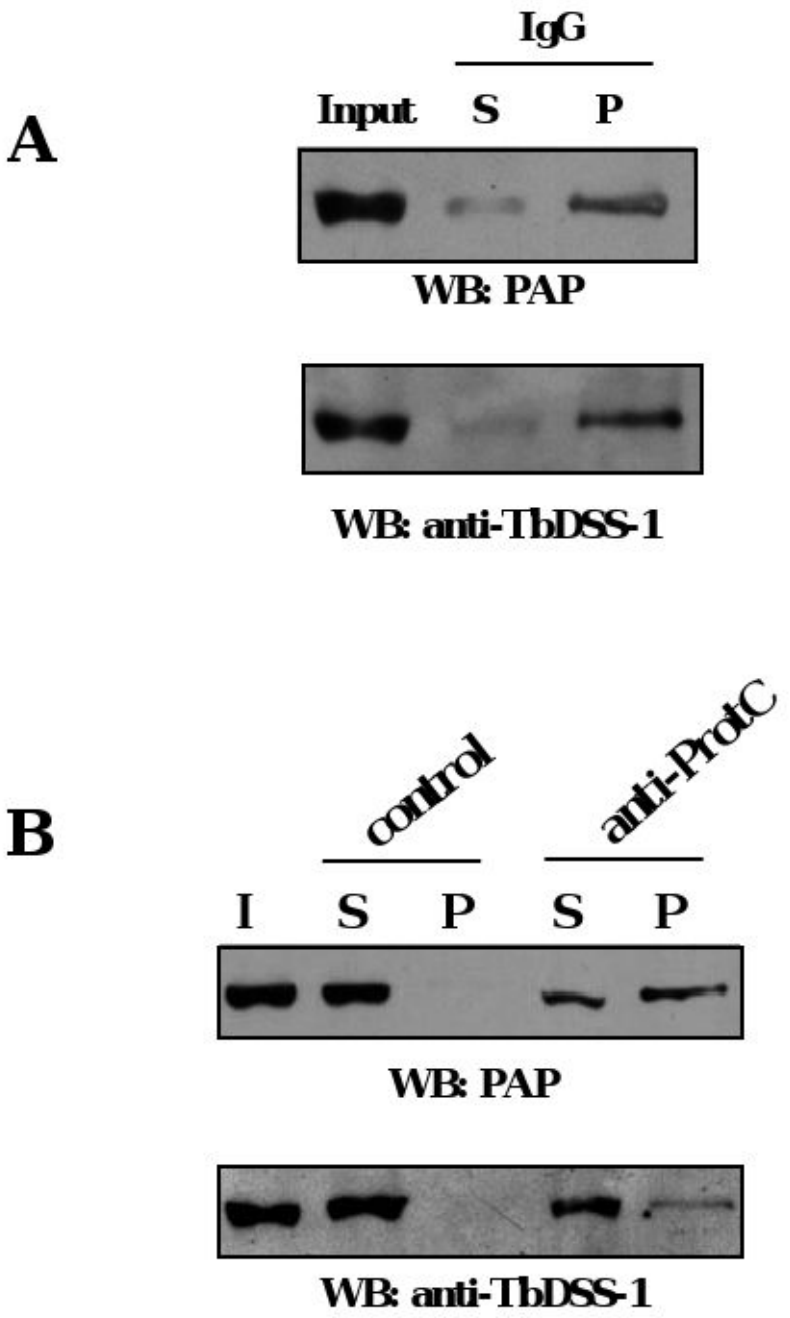

Fig. 4.

In vivo interaction between TbSUV3 and TbDSS-1. (A) Peak glycerol gradient fractions from TbSUV3-PTP expressing cells were pooled and incubated with IgG Sepharose beads. Beads were washed, and ten percent of input (I), supernatant (S), and pellet (P) fractions were analyzed by SDS-PAGE and western blot with the PAP reagent (top) and anti-TbDSS-1 (bottom). (B) TbSUV3-PTP was isolated from pooled glycerol gradient fractions by immunoprecipitation with anti-ProtC antibodies. Reactions lacking primary antibodies (control) were performed as a negative control. Ten percent of input (I), supernatant (S), and pellet (P) fractions were analyzed by western blot as in panel A.