FEBS Letters 583 (2009) 2333–2338

FEBS Letters

journal homepage: www.FEBSLetters.org

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

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ARTICLE INFO

Article history:
Received 29 May 2009
Accepted 11 June 2009
Available online 18 June 2009

Edited by Michael Ibba

Keywords:
RNA turnover
RNA processing
Trypanosome
RNA helicase
Exoribonuclease

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.

Structured summary:
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 co-immunoprecipitation* (MI:0007)

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 known exoribonuclease involved in yeast mitochondrial RNA (mtRNA) turnover [8]. *Saccharomyces 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

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0014-5793/$36.00
doi:10.1016/j.febslet.2009.06.024

[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′-GCATCGATGCTATTATTAGA ACAGTTTCTGTACTATATTG -3′), SUV3-8 (5′-GCGGATCCAACGCCGC GTGAGTCTTCC-3′), SUV3 -9 (5′-GCGGATCCGTGCCTTCGGGTACCA GTC-3′).

2.2. Trypanosome cell culture, transfection, and cell fractionation

The procyclic form (PF) *T. 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′-GCCGGGGCC CAAGACCTCAGGTGTGGTGCC-3′) forward and TbSUV3-PTP3′ (ATA-AGAATGCGGCCGCGGCAACCTCCGCAACAGCTC-3′) reverse primers and cloned into the ApaI/Not I 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 20 μg 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 [20 mM Tris–HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 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 microliters 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 [20 mM Tris–HCl (pH 7.7), 150 mM KCl, 3 mM $MgCl_2$, 0.5 mM DTT, 0.1% Tween 20[and resuspended in Buffer A [10 mM Tris–HCl (pH 8.0), 25 mM KCl, 10 mM $MgCl_2$]. 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 micrograms 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 microliters of Protein G Sepharose beads was 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 [20 mM Tris–HCl (pH 7.7), 150 mM KCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% Tween 20], 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