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Identification and characterization of nuclear non-canonical poly(A) polymerases from *Trypanosoma brucei*

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Abstract

Regulation of nuclear genome expression in *Trypanosoma brucei* is critical for this protozoan parasite's successful transition between its vertebrate and invertebrate host environments. The canonical eukaryotic circuits such as modulation of transcription initiation, mRNA splicing and polyadenylation appear to be nearly non-existent in *T. brucei* suggesting that the transcriptome is primarily defined by mRNA turnover. Our previous work has highlighted sequence similarities between terminal RNA uridylyl transferases (TUTases) and non-canonical poly(A) polymerases, which are widely implicated in regulating nuclear, cytoplasmic and organellar RNA decay throughout the eukaryotic lineage. Here, we have continued characterization of TUTase-like proteins in *T. brucei* and identified two nuclear non-canonical poly(A) polymerases (ncPAPs). The 82 kDa TbncPAP1 is essential for viability of procyclic and bloodstream forms of *T. brucei*. Similar to Trf4/5 proteins from budding yeast, TbncPAP1 requires protein cofactor(s) to exert poly(A) polymerase activity *in vitro*. The recombinant 54 kDa TbncPAP2 showed a PAP activity as an individual polypeptide. Proteomic analysis of the TbncPAP1 interactions demonstrated its association with Mtr4 RNA helicase and several RNA binding proteins, including a potential ortholog of Air1p/2p proteins, which indicates the presence of a stable TRAMP-like complex in trypanosomes. Our findings suggest that TbncPAP1 may be a "quality control" nuclear PAP involved in targeting aberrant or anti-sense transcripts for degradation by the 3'-exosome. Such mechanisms are likely to play a major role in alleviating promiscuity of the transcriptional machinery.

Keywords

Trypanosoma brucei; Non-canonical poly(A) polymerase; TUTase; RNA decay; TRAMP; MTR4; Exosome

1. Introduction

The eukaryotic transcriptome is defined by the concerted action of a variety of protein complexes carrying out transcription, capping, splicing, polyadenylation and subsequent nuclear export and degradation of RNA. The intricate levels of regulation built into each process allow the organism to quickly respond to changes in environmental conditions. The

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2008.11.004.

protozoan parasite *Trypanosoma brucei* spp. undergoes radical transformation with respect to cell surface protein expression, metabolism, and morphology as it transitions between its arthropod and vertebrate hosts. Surprisingly, many of the universal mechanisms regulating eukaryotic gene expression appear to be all but absent in these organisms. Transcription initiation, typically a focal point of RNA regulation, does not play a significant role in *T. brucei* [1] and few transcription factors are encoded in the trypanosome genome [2]. With the exception of the spliced leader (SL) RNA, there are no known RNA polymerase II (Pol II) promoters and transcripts are initially made in multicistronic units composed of seemingly unrelated genes. Individual messages are then generated by 5'-*trans* splicing, which adds a universal pre-capped spliced leader RNA to all mRNAs, and 3'-polyadenylation [3].

Because the efficiency of *trans* splicing for the downstream message and polyadenylation of the corresponding upstream transcript rely on a shared polypyrimidine tract, the regulation of individual transcript abundance at this stage of processing does not appear to play a major role [4]. As such, RNA binding factors recognizing specific sequence elements within 3'-UTRs and thus affecting the abundance of individual mRNAs came into focus (for review see [5]). Indeed, sequencing of trypanosomal genomes has revealed the presence of a highly expanded family of RNA binding proteins likely to be involved in regulating RNA levels [6]. For example, the RRM-domain containing proteins TcUBP1 and TcUBP2 have been shown to interact with the 3'-UTRs of mRNAs containing AU-rich elements (ARE). This interaction results in the stage-specific destabilization of these mRNAs in *T. cruzi* [5,7].

The 3'-end mRNA polyadenylation by the canonical nuclear poly(A) polymerase (PAP) is a virtually universal processing step leading to mRNA stabilization, export and translation. Trypanosomal PAP (Tb927.3.3160) was identified via homology to other eukaryotic PAPs [8]. The second putative canonical PAP (Tb927.7.3780) is readily detectable by Blast searches of GeneDB database (<http://www.genedb.org/>). Although specific roles of these proteins in *T. brucei* have not been characterized, the high degree of sequence similarity suggests functions alike to other eukaryotic nuclear PAPs. Recently it has become evident that nuclear polyadenylation is not limited to mRNA 3'-end processing. In budding yeast, for example, polyadenylation of non-coding, misfolded or hypermodified RNAs by the Trf4p/Air2p/Mtr4p polyadenylation (TRAMP) complex results in recruitment of the nuclear exosome and consequent RNA degradation (reviewed in [9]). The catalytic subunit of the TRAMP complex, Trf4p, requires an RNA binding zinc-knuckle protein Air2p for enzymatic activity. We have noted previously that Kinetoplastida genomes encode a family of RNA nucleotidyl transferases [10] which includes the mitochondrial RNA editing [11,12] and cytosolic [13,14] terminal uridylyl transferases (TUTases), and several proteins of unknown nucleotide substrate specificity. Identification of trypanosomal mitochondrial poly(A) polymerase KPAP1 [15] via homology to TUTases further illustrated the high degree of sequence similarity between TUTases and non-canonical poly(A) polymerases.

Here we report the identification and characterization of two nuclear non-canonical PAPs, TbncPAP1 and 2, in *Trypanosoma brucei*. Both proteins are localized to the nucleus with TbncPAP1 being essential for viability of the parasite's procyclic and blood-stream forms. Recombinant TbncPAP1 purified from *Escherichia coli* was inactive while its native complex purified from *T. brucei* possessed PAP activity. In contrast, TbncPAP2 displayed a processive polyadenylation activity as a recombinant polypeptide isolated from bacteria and *T. brucei*. Proteomic analysis of the TbncPAP1 complex has revealed an association with the trypanosomal Mtr4 and putative Air1p ortholog [16]. Cross-TAP purification of the *T. brucei* Mtr4 complex confirmed an association with TbncPAP1, suggesting the presence of a stable TRAMP-like complex in trypanosomes. The plurality of RNA binding proteins in this complex, as compared to TRAMP from budding yeast [17–19], may indicate a significant divergence of RNA substrates targeted by TbncPAP1.

2. Materials and methods

2.1. Plasmids construction and mutagenesis

Plasmids for inducible RNAi expression were generated by cloning PCR-amplified (primers: TbncPAP1 A374–A375, Tbnc-PAP2 A453–A454) ~500 bp gene fragments into the p2T7-177 vector [20]. Constructs for tetracycline-inducible expression of C-terminally TAP tagged fusion proteins were generated by cloning PCR-amplified genes (primers: TbncPAP1 A264–A265, TbncPAP2 A457–A458) into the pLEW79-MH-TAP vector (kind gift of Marylyn Parsons, SBRI). For expression in *E. coli*, full length TbncPAP1 and 2 genes (primers: TbncPAP1 A127–A128, TbncPAP2 A451–A452) were cloned into the pET-15b vector (Novagen) to generate an N-terminal 6×His tagged proteins. A plasmid for expression of N-terminally eYFP-tagged TbncPAP2 was generated by cloning the eYFP and TbncPAP2 genes (primers: TbncPAP2 A710–A452, EYFP A708–A709) into the pLEW100 vector [21]. Mutations (primers: TbncPAP1 A390–A391, TbncPAP2 A530–A531) were introduced with the QuickChange II XL mutagenesis kit (Stratagene). Oligonucleotides used in this study are listed in Table S1.

2.2. Protein expression and purification

Expression of ncPAP1 or 2 was induced with 1 mM IPTG for 4 h at 6°C in a 1 L of BL21(DE) RIL *E. coli* strain grown in 2YT media. Cells were collected, resuspended in 25 mL of lysis buffer (50 mM HEPES (pH 8.0), 50 mM NaCl, 0.1 mg/mL lysozyme) and lysed in a French pressure cell. Extracts were cleared by centrifugation for 90 min at $165,000 \times g$ at 4 °C for 1 h and loaded onto a 3 mL column with Talon (Clontech) metal affinity resin. Column was washed with 10 mM imidazole and 6His-tagged proteins were eluted with 200 mM imidazole. Final fraction was diluted 4-fold with 50 mM HEPES (pH 7.5), 1 mM DTT, 0.1 mM EDTA, loaded on a 1 mL HiTRAP SP Column (GE) and eluted with a gradient of KCl from 50 to 500 mM. For purification of TAP-tagged proteins from *T. brucei*, cells were grown to $\sim 2 \times 10^7$ cells/mL in SDM-79 media with 10% of serum, harvested and washed in PBS. Cell pellet was resuspended in 10mL of extraction buffer (50 mM Tris–HCl (pH 8.0), 150 mM KCl, 2 mM EDTA, 0.1% NP-40, and 1/2 tablet of Complete protease inhibitor (Roche)). The extract was sonicated 4×15 s at 2.5 W with 5 min intervals and cleared at $165,000 \times g$ at 4 °C for 1 h. Tandem affinity purifications from were performed as described [22]; KCl was maintained at 150 mM.

2.3. Mass spectrometry

Fractions from calmodulin column were mixed with 1/5 volume of 100% trichloric acid/0.5% deoxycholate, incubated on ice for 30 min, and centrifuged for 30 min at $18,000 \times g$ at 4 °C. The pellet was washed with ice-cold acetone and air-dried. For the total digest, a two-step protocol with LysC and trypsin proteases was carried out. The pellet was re-suspended in 25 µl of LysC buffer (8 M urea, 100 mM Tris–HCl (pH 8.5)), LysC added to 1:100 mass ratio and incubated at 37 °C for 4 h. For trypsin digestion, the mixture was supplemented with 75 µl of 50 mM NH_4HCO_3 (pH 7.8), trypsin added to mass ratio 1:100, and digestion continued overnight. Peptides were purified using Vivapure C18 microspin columns (Vivascience Corp.). Eluted samples were reduced by vacuum evaporation to ~1 µl and 10 µl of 2% ACN+ 0.1% formic acid was added. 0.5–1 µl aliquots were subjected to LC–MS/MS analysis using an LTQ mass spectrometer running in positive ion mode via 100 µm ID C18 nanospray tips. Standard formic acid/water/acetonitrile gradients were employed. The instrument's "BioWorks Browser" was used for peak analysis and DB searching via both Sequest and Mascot search engines. The database for Mascot searches comprised the translated *T. brucei* genome with built-in decoy searching against the corresponding randomized library. For Sequest, the subset of UniProt entries possessing relevant header info. The presence and numbers of peptides with Mascot expect score ≤ 0.05 were considered candidate hits.

2.4. Parasite cell culture and RNA interference

Procyclic *T. brucei* were maintained in SDM-79 media at 27 °C. Bloodstream form cells were cultured in HMI-9 media at 37 °C in 5% CO₂. The expression constructs were transfected into procyclic 29–13 or a “single-marker” bloodstream *T. brucei* strains [21] followed by limiting dilution clonal selection of phleomycin-resistant cell lines. RNAi was performed as described in [23]. Cell counts were taken daily to attain growth curves.

2.5. Immunofluorescence and YFP imaging

T. brucei cells expressing an N-terminal YFP-TbncPAP2 fusion were immobilized with 4% formaldehyde in PBS containing DAPI and mounted on poly-Lysine coated microscope slides. Images were taken with a Zeiss 200 M inverted microscope utilizing filters and excitation sources for YFP and DAPI.

2.6. Sub-cellular fractionation and Western blotting

Nuclei from procyclic form *T. brucei* cells were isolated according to [24]. The gradient centrifugation time was increased to 4 h to achieve greater separation. Mitochondrial fraction was isolated as described in [25]. Polyclonal rabbit antibodies to TbncPAP1 and 2 were generated at Covance Inc., and purified by antigen affinity chromatography. Chemiluminescent images were acquired on a Fuji LAS-4000 digital reader.

2.7. Enzymatic activity assays

Activity assays were performed at 100 μM of nucleotide triphosphates, 100 nM of RNA substrate in a 10 μl reaction at 27 °C. The optimal reaction conditions for TbncPAP1 and 2 were determined to be 50 mM HEPES (pH 8.0), 10 mM MgOAc, 1 mM DTT and 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM MgOAc, 1 mM DTT, respectively. The 6[A] RNA (GCUAUGUCUGUCAACUUGAAAAAA) substrate was purchased from Dharmacon Inc., purified on 15% acrylamide/urea gel. Reactions were stopped with two volumes of 5 mM EDTA, 95% (v/v) formamide. Products were resolved on a 15% (w/v) acrylamide/8M gel and exposed to a phosphor storage screen. For determining the kinetic parameters, a 60 μl reactions containing [α -³²P]ATP (1–100 μM, ~10,000–500 cpm/pmol) and 10 nM of purified TbncPAP2 were set up, and 10 μl samples were taken at 0.5, 1, 2, 4, and 8 min. Samples were processed as described for filter-based assay in [22]. The apparent K_m and catalytic rate k_{cat} for ATP incorporation were obtained by fitting the initial velocities as a function of ATP concentration from three experiments into a standard Michaelis–Menten kinetics model. The Sigma Plot Enzyme Kinetics software package was used for calculations of K_m , V_{max} , and standard deviations.

3. Results

3.1. Identification of TbncPAP1 and 2

Protein sequences of RET1, RET2, TUT3, and TUT4 TUTases [26] and non-canonical poly (A) polymerases from fission and budding yeast, *C. elegans* and *X. laevis* (reviewed in [27]) were used in iterative Blast searches to define a family of TUTase-like proteins in *T. brucei* (Fig. 1). The domain organization of TbncPAP1 and 2 resembles that of TUT4 [13,14] and KPAP2 [15,28] pointing out a lack of the middle domain. The protein sequences of the middle domain are divergent among enzymes shown in Fig. 1, but the positioning of the middle domain as an “insert” within the N-terminal domain of TUTases [11,29–31] and the mitochondrial PAP, KPAP1 [15], is preserved. The insertion site represents a short loop connecting two β -sheets in an otherwise highly conserved catalytic domain common to all members of the DNA polymerase beta (Pol β) superfamily [32]. By analogy to TUTases [13,31], the ncPAP's NTD and CTD are likely to form a compact bi-domain while their N-terminal and C-terminal

extensions show no appreciable similarities except to homologous sequences in other Trypanosomatids. Multiple sequence alignments illustrate that amino acid residues responsible for UTP binding and catalysis in RET1 [30], RET2 [31] and TUT4 [13] are mostly invariant among TUTases and non-canonical PAPs (Fig. 2). Therefore, in contrast to canonical PAPs, sequence-based predictions of UTP/ATP specificity within TUTase-ncPAP family are unlikely to be accurate; the correct annotations of their cellular localization, functions and enzymatic identities require experimental analysis.

3.2. TbncPAP1 requires additional factors for enzymatic activity

The full-length *TbncPAP1* gene was expressed in *E. coli* and purified to apparent homogeneity by sequential metal affinity and cation exchange chromatography. The recombinant protein was soluble during expression and purification, as monitored by Western blotting with anti-His tag antibodies, but apparently inactive in the nucleotidyl transfer assays described in Section 2 (data not shown). To determine if additional subunits are required for enzymatic activity, TbncPAP1 was expressed as a C-terminally TAP-tagged protein [33] and purified via tandem affinity chromatography from total cell lysates of procyclic *T. brucei*. As a control, a mutated protein carrying D381A and D383A substitutions in the catalytic metal binding residues was purified likewise (Fig. 3A). The TbncPAP1 complex was active indicating a requirement for co-purifying protein factor(s) for poly(A) polymerase activity. The mutated protein was inactive as expected (Fig. 3B), while there were no discernible differences in the protein profile on SDS gel (Fig. 3A). TbncPAP1 complex exhibited a preference for ATP, and increasing ATP concentration in the reaction led to a net primer extension of 20–25 nucleosides during the reaction (Fig. 3C).

3.3. Recombinant TbncPAP2 is a highly processive PAP

Unlike TbncPAP1, recombinant TbncPAP2 purified from *E. coli* (Fig. 4A), was active and displayed a preference for ATP (Fig. 4B). As a control for possible contamination with bacterial PAP, a D171A mutation was introduced into the active site and the protein was purified by the same procedure (Fig. 4A). The mutated protein was inactive confirming polymerase activity of TbncPAP2. At enzyme concentrations exceeding 10 nM, polymerization products reached several hundred nucleosides in length during a 30 min reaction (Fig. 4C). The catalytic parameters for TncPAP2 were determined by filter assay as described for RET1 [22]. The apparent K_m for ATP of $2.6 \pm 3.2 \mu\text{M}$ and k_{cat} of $96.4 \pm 19.8 \text{ min}^{-1}$ indicates that TbncPAP2's catalytic efficiency exceeds that of canonical PAPs by 100–500 fold [34]. Because interacting factors may modulate nucleotide specificity of non-canonical PAPs [35,36], we next analyzed the NTP specificity of TbncPAP2 purified from *T. brucei* (Fig. 4D, left panel). Although the elongation products were limited to ~10 nt (Fig. 4D, right panel), most likely due to low enzyme concentration in the reaction mixture, the NTP specificities were virtually identical between the individual protein and the native TbncPAP2 complex.

3.4. TbncPAP1 and TbncPAP2 are localized to the nucleus

Western blotting of *T. brucei* sub-cellular fractions demonstrated that TbncPAP1 and TbncPAP2 are localized in the nucleus. As shown in Fig. 5A, TbncPAP1 and TbncPAP2 were enriched in the density gradient purified nuclear fraction. The nuclear transcription factor TFIIB [37] showed a similar distribution. Immunoblotting for the cytosolic protein HSP70.4 was used to monitor separation of the cytosolic, nuclear and mitochondrial fractions. To confirm nuclear localization of TbncPAP1, live procyclic *T. brucei* cells were fixed, permeabilized, treated with antigen-purified antibodies to Tbnc-PAP1 and analyzed by immunofluorescence microscopy (Fig. 5 B, top panels). Staining of cell nuclei with DAPI confirmed the nuclear localization of this protein. To address the localization of TbncPAP2, a stable cell line expressing TbncPAP2 as an N-terminal fusion with enhanced yellow fluorescent

protein (eYFP) was generated. Counterstaining with DAPI confirmed the nuclear localization of this protein. Thus, both ncPAP1 and ncPAP2 are localized in the nucleus.

3.5. TbncPAP1 is essential for parasite viability and expressed at comparable levels in procyclic and bloodstream life cycle stages

To determine the effect of TbncPAP1 repression on cell viability, an inducible RNAi knockdown was performed. Depletion of TbncPAP1 in procyclic *T. brucei* resulted in severe growth inhibition beginning at 48 h post-RNAi induction (Fig. 6A). Western blot analysis of TbncPAP1 protein levels revealed a loss of ~80% by 24 h and a decline beyond sensitivity of the assay after 48 h (Fig. 6B). A comparable growth phenotype was observed in bloodstream form parasites (not shown) indicating that TbncPAP1 is essential in both life cycle stages. To assess the expression levels of TbncPAP1 in bloodstream and procyclic forms, equal number of parasites were harvested at 0.5 and 5×10^6 cells/ml, respectively, and separated by SDS PAGE. Western blotting demonstrated similar protein levels in both forms (Fig. 6C). Combined with apparently equal relative abundance of TbncPAP1 mRNA, as determined by quantitative RTPCR in reference to α -tubulin mRNA (not shown), this suggests equal levels of *TbncPAP1* gene expression in both life cycle stages. RNAi-induced knockdown of TbncPAP2 produced no discernable changes in growth kinetics of procyclic (Fig. 6D) or bloodstream form parasites (not shown). Western blotting analysis of TbncPAP2 knockdown after 72 h of RNAi induction demonstrated a virtually complete protein depletion suggesting that the gene is not essential under the experimental cell culture conditions.

3.6. TbncPAP1 is associated with Mtr4 RNA helicase and a putative RNA binding subunit of the TRAMP complex

To place TbncPAP1 into a possible functional context, we next identified major components co-purifying with a TAP-tagged protein (Fig. 3A). The final calmodulin fraction was precipitated, digested with LysC and trypsin and analyzed by LC-MS/MS mass spectrometry. The trypanosomal Mtr4 RNA helicase [16] and several hypothetical RNA binding proteins were detected with high confidence (Table 1 and Table 2). Of particular interest, the Tb11.02.4470 zinc-knuckle protein was one of the highest scoring polypeptide making it a strong candidate for an ortholog of the Air1p or Air2p, proteins required for the catalytic activity of yeast “quality control” PAPs [18]. At the protein sequence level, Tb11.02.4470 and yeast Air1p display 13% identity and 22% similarity mostly limited to a zinc-knuckle motif (Supplementary Fig. 1). It should be noted that a different hypothetical protein, Tb11.01.1270, produced the highest scores in Blast searches of the *T. brucei* database (<http://www.genedb.org>) with Air1p or Air2p from *S. cerevisiae*. However, neither Tb11.01.1270 nor canonical PAP1 [8] and 2 [Tb927.7.3780], or subunits of the nuclear 3'-exosome [38,39] were detected in our analysis of TbncPAP1 fraction. Affinity purification of TbncPAP2 proved to be too inefficient to generate material allowing confident mass spectrometric detection of any protein except the bait and common contaminants such as those identified for TbncPAP1 (Table 2). Mass spectrometry and Western blotting analysis of the ncPAP1 TAP fraction provided no evidence for the presence of ncPAP1 and 2 in the same particle (not shown).

To verify a stable association between TbncPAP1 and Mtr4, a cross-purification was performed with C-terminally TAP-tagged Mtr4 under identical conditions (Fig. 7A, SDS PAGE). Western blotting confirmed the presence of TbncPAP1, but not TbncPAP2, in the Mtr4 complex, further demonstrating distinct interaction patterns for the two non-canonical PAPs. Finally, to validate the presence of an enzymatically active TRAMP complex in *T. brucei*, a nucleotidyl transferase assay was performed with the Mtr4-purified fraction. In the presence of a synthetic 5'-labeled 6[A] RNA substrate and increasing concentrations of ATP, a PAP activity was detected (Fig.

7B). The differences in elongation patterns observed between Fig. 3C and Fig. 7B are most probably caused by lower TbncPAP1 amount in the latter reactions.

Discussion

Responding to changing environments is essential for the multihost life cycle parasites such as Trypanosomes. However, transcription of the majority of protein-coding genes is essentially unregulated and available evidence points to predominantly posttranscriptional mechanisms of gene expression in these organisms. The pathways appear to be in place to regulate the abundance of individual transcripts through “readout” of specific sequences in the 3'-UTR by RNA binding proteins ultimately leading to the differential stability of mRNA species. Studies in budding yeast revealed a novel mechanism of aberrant and non-coding RNA degradation triggered by polyadenylation and executed by the 3'-nuclear exosome. Such polyadenylation is accomplished by non-canonical poly(A) polymerases, members of the DNA polymerase β -superfamily which are most closely related to RNA uridylyl transferases [26]. It appears plausible that in an organism lacking many of the “canonical” regulatory mechanisms, such as trypanosomes, the polyadenylation-triggered nuclear degradation may be responsible for a much larger repertoire of functions. A homolog of the yeast Mtr4p helicase was reported in *T. brucei* but no indication of the TRAMP-like complex was found [16].

In this work we report identification of two trypanosomal non-canonical poly(A) polymerases termed TbncPAP1 and TbncPAP2. The possibility of TbncPAP1 (Tb927.8.1090) being an homolog of yeast Trf4p/Pap2p has been proposed earlier and the protein was termed nuclear-poly(A)-polymerase-like (NPALP) by Cristodero and Clayton [16]. Here we present evidence that a stable TRAMP-like complex does exist in *T. brucei* and its catalytic subunit, TbncPAP1, is essential for viability of procyclic and bloodstream form parasites. While TbncPAP1 purified from bacteria was inactive, its native complex possessed a poly(A) polymerase activity producing short (20–25 nt) A-tail, a pattern remarkably similar to the one generated by yeast's TRAMP. Hypothetically, the requirement for an RNA binding subunit may be fulfilled by any of the four major RNA binding proteins identified in the TbncPAP1 complex. The full characteristics of this complex remains to be determined and functions of each polypeptide characterized. Based on its abundance in the TbncPAP1 complex and similarity searches we propose that the zinc-knuckle protein Tb11.02.4470 is a variant of the yeast Air1p/2p proteins. In yeast, at least two TRAMP complexes exist that differ by catalytic subunits (Trf4 or 5) and RNA binding factors (Air1 or 2) while Mtr4 is a shared component [9]. Genes with duplicated function are common in *S. cerevisiae* due to a genome duplication [40] whereas trypanosomes apparently have only one copy of catalytic (ncPAP1) and RNA binding (Tb11.02.4470) subunits.

In addition, TbncPAP1 associates with the trypanosomal Mtr4 helicase. In yeast, Mtr4 interacts with Trf4 recruiting the nuclear exosome to polyadenylated transcripts and thus triggering degradation. We confirmed, through a cross-TAP purification of TbMtr4, that a stable association exists between this protein and TbncPAP1 and that the purified complex retains polyadenylation activity. Although RNA targets of TbncPAP1 remain to be elucidated, it is appealing to consider a role for the trypanosomal TRAMP complex in the degradation of RNAs produced by seemingly unregulated transcription, particularly molecules which are anti-sense to mRNAs and may trigger RNAi. In addition, targeted RNAs may include SL RNAs [41] and rRNA precursors [42] as they have been shown to undergo polyadenylation in Trypanosomatids. The list of RNA binding proteins associated with TbncPAP1, in addition to the Air1p-like polypeptide, may provide insight into the broader functions of this complex. Each RNA-binding protein may, for example, serve as the bridge between TbncPAP1 and distinct classes of RNAs.

While TbncPAP2 is not essential in procyclic or bloodstream form parasites, its involvement in regulation of gene expression is still possible under certain environmental conditions. The exact role of TbncPAP2 has yet to be elucidated but most likely will be distinct from that of TbncPAP1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

UTR	untranslated region
ARE	A/U rich elements
TRAMP	Trf4p/Air2p/Mtr4p polyadenylation complex
SL	spliced leader RNA
Pol II	RNA polymerase II
PAP	poly(A) polymerase
ncPAP	non-canonical poly(A) polymerase
TUTase	terminal RNA uridylyl transferase

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Fig. 1.

Domain organization of the TUTases-ncPAPs protein family in *T. brucei*. Domain boundaries were determined based on crystal structures of RET2 [31] and TUT4 [13], and multiple sequence alignments shown in Fig. 2.

Fig. 2.

Partial multiple sequence alignment of TUTases and non-canonical poly(A) polymerases. Sequence alignments were performed with the T-coffee algorithm and refined manually. Protein sequences used in the alignment are: Sc TRF5p (AAC49099), Sp TRF14 (NP_001018181), Ce Gld-2 (NP_491842), Tb KPAP1 (ABV66277), Sp CID1 (NP_594901), Tb RET1 (AAK38334), Tb RET2 (AAO63567), Tb TUT3 (XP_822966) and Tb TUT4 (DQ923393). Signature motif of the Pol β nucleotidyl transferase superfamily is boxed. The N-terminal (NTD) and C-terminal (CTD) domains are shown. Identical amino acids are white on black; blocks of similar amino acids are black on grey background. Amino acids participating in UTP binding by TUTases are indicated by asterisks; RNA binding contacts in TUT4 are shown by black circles. Metal-coordinating carboxylate residues are shown by arrows.

Fig. 3. Purification of the active TbncPAP1. (A) Tandem affinity purification of wild type and catalytically inactive TbncPAP1. M: molecular mass marker. Proteins were separated on 8–16% gradient SDS-PAGE and visualized with SYPRO Ruby stain. (B) NTP specificity of TbncPAP1. The 5'-labeled 6[A] RNA substrate was incubated with TAP-purified TbncPAP1 in the presence of different nucleotide triphosphates. The double mutant (D381A, D383A) was tested to confirm the enzymatic identity of TbncPAP1. c: RNA substrate; +e: RNA substrate was incubated with protein in the absence of NTPs. (C) Processivity of TbncPAP1 *in vitro*. Reaction was carried out for 30 min with 1, 10 or 100 μ M of ATP. The products were resolved on a 15% polyacrylamide/8 M urea gel.

Fig. 4.

Purification and enzymatic activity of TbncPAP2. (A) Full-length TbncPAP2 and a protein containing a D171A substitution in the active site were expressed in *E. coli* as N-terminally 6His-tagged fusions, and purified by sequential metal affinity and anion exchange chromatography. Proteins were separated on 8–16% gradient SDS-PAGE and visualized with SYPRO Ruby stain. (B) NTP specificity of TbncPAP2. The 5'-labeled 6[A] RNA substrate was incubated with TbncPAP1 in the presence of 100 μ M of nucleotide triphosphates for 30min. The mutant D171A was tested to preclude the possibility of bacterial PAP contamination. c: RNA substrate; +e: RNA substrate was incubated with protein in the absence of NTPs. The products were resolved on a 15% polyacrylamide/8M urea gel. (C) Processivity of TbncPAP2. Assay was carried out as in (B), with varied amounts of TbncPAP2 in the presence of 100 μ M of ATP. (D) Right panel: tandem affinity purification of wild-type TbncPAP2 from *T. brucei*. M: molecular mass marker. Proteins were separated on 8–16% gradient SDS-PAGE and visualized with SYPRO Ruby stain. Left panel: NTP specificity of TAP-purified TbncPAP2. Assay was carried out and visualized as in (B).

Fig. 5.

Cellular localization of TbncPAP1 and 2. (A) Sub-cellular fractionation of *T. brucei* cells. Nuclei and mitochondria were purified by density gradient from 2 L of PF cultures grown to $\sim 10^7$ cells/ml. Supernatant obtained after hypotonic cell lysis and removal of the membrane fraction by centrifugation served as source for cytosolic proteins. Western blots were probed with antibodies for TbncPAP1 and 2. A non-specific band of ~ 50 kDa is often observed cross-reacting with rabbit polyclonal antibodies in the mitochondrial extracts. Antibodies for TFIIB and Hsp70.4 were utilized to monitor fractionation. After transfer, the SDS-PAGE gel was stained with Coomassie blue to ensure equal loading. (B) Analysis of sub-cellular localization by fluorescence microscopy. TbncPAP1: wild type procyclic *T. brucei* cells were mounted and permeabilized with Triton X-100. Cells were then exposed to affinity purified polyclonal antibody followed by secondary antibody conjugated to FITC. TbncPAP2: cells expressing an N-terminal EYFP-TbncPAP2 fusion protein were immobilized and mounted on slides. Images were taken in the presence of DAPI to visualize DNA.

Fig. 6.

RNAi-mediated inhibition of ncPAPs expression. (A) RNAi knockdown of TbncPAP1. RNAi was induced in a clonal PF *T. brucei* line derived from 29–13 [21] cells by transecting a p2T7-177-based [20] construct for expressing a double-stranded RNA. Circles—10 µg/ml of tetracycline was added to the media, diamonds—mock induction. (B) Depletion of TbncPAP1 protein following RNAi knockdown. Samples were taken at each time point and analyzed on immunoblots. C. Western blotting of TbncPAP1 in procyclic form (29–13 cell line) and bloodstream form (“single marker”). Membrane was probed with anti-β-tubulin antibodies and gel stained with Coomassie to demonstrate equal protein loading. (D) RNAi knockdown of TbncPAP2 was carried out as in (A). Loss of TbncPAP2 protein was examined after 72 h of RNAi induction.

Fig. 7.

Association of TbncPAP1 with Mtr4 RNA helicase. (A) Tandem affinity purification of Mtr4 from *T. brucei*. M: molecular mass marker. Proteins were separated on 8–16% gradient SDS-PAGE and visualized with SYPRO Ruby stain. Western blotting was performed by sequential treatment of the same membrane with antibodies against calmodulin binding peptide (CBP), which detects a tagged Mtr4, TbncPAP1 and 2, Hsp70.4. (B) Poly(A) polymerase activity of the TAP-purified Mtr4 complex. The 5'-labeled 6[A] RNA substrate was incubated with Mtr4 fraction in the presence of 1, 10, and 100 μ M of ATP. c: 6[A] RNA substrate; +e: RNA substrate with protein, lacking ATP. The products were resolved on a 15% polyacrylamide/8 M urea gel.

Table 1

Proteins identified in tandem affinity purified TbncPAP1 complex. Number of unique peptides matches with e-value below 0.05 and scores obtained in Mascot searches are shown.

Protein	Pfam motif	Peptide #	Score
Tb927.8.1090 (TbncPAP1)	NTP_transf_2	15	490
Tb11.02.4470 (Air1p/2p)	Zinc knuckle (CCHC)	14	601
Tb10.6k15.3220 (Mtr4)	DEAD/DEAH helicase	13	305
Tb09.211.2150 (PABP1)	PABP, RRM	9	208
Tb09.211.4540	RRM	9	184
Tb10.61.2130	DEAD/DEAH helicase	7	137
Tb11.02.4570	Pumilio RNA binding	5	202
Tb927.2.4710	Zinc knuckle, RRM	3	207

Table 2

Contaminating proteins in TbncPAP1 TAP-purified fractions. Polypeptides which produced number of unique peptides and scores comparable to those listed in Table 1.

Protein	Peptide #	Score
β -tubulin, Tb927.1.2330	19	838
α -tubulin, Tb927.1.2340	12	1092
Elongation factor 1 α , Tb10.70.5650	12	549
Pyruvate phosphate dikinase, Tb11.02.4150	9	304
Glutamate dehydrogenase, Tb09.160.4310	8	190
Importin α subunit, Tb927.6.2640	7	290
Retrotransposon hot spot, Tb927.2.400	5	167