TbRGG1, an essential protein involved in kinetoplastid RNA metabolism that is associated with a novel multiprotein complex

Hassan Hashimi, Alena Zíková, Aswini K. Panigrahi, Kenneth D. Stuart and Julius Lukes

RNA 2008 14: 970-980; originally published online Mar 27, 2008;
Access the most recent version at doi:10.1261/rna.888808

TbRGG1, an essential protein involved in kinetoplastid RNA metabolism that is associated with a novel multiprotein complex

HASSAN HASHIMI,[1] ALENA ZÍKOVÁ,[2] ASWINI K. PANIGRAHI,[2] KENNETH D. STUART,[2] and JULIUS LUKEŠ[1]

[1]Biology Centre, Institute of Parasitology, Czech Academy of Sciences, and Faculty of Sciences, University of South Bohemia, České Budějovice (Budweis), Czech Republic
[2]Seattle Biomedical Research Institute, Seattle, Washington 98109, USA

ABSTRACT

The uridine insertion/deletion RNA editing of kinetoplastid mitochondrial transcripts is performed by complex machinery involving a number of proteins and multiple protein complexes. Here we describe the effect of silencing of *TbRGG1* gene by RNA interference on RNA editing in procyclic stage of *Trypanosoma brucei*. TbRGG1 is an essential protein for cell growth, the absence of which results in an overall decline of edited mRNAs, while the levels of never-edited RNAs remain unaltered. Repression of TbRGG1 expression has no effect on the 20S editosome and MRP1/2 complex. TAP-tag purification of TbRGG1 coisolated a novel multiprotein complex, and its association was further verified by TAP-tag analyses of two other components of the complex. TbRGG1 interaction with this complex appears to be mediated by RNA. Our results suggest that the TbRGG1 protein functions in stabilizing edited RNAs or editing efficiency and that the associated novel complex may have a role in mitochondrial RNA metabolism. We provisionally name it putative mitochondrial RNA-binding complex 1 (put-MRB complex 1).

Keywords: RNA editing; mitochondrion; trypanosome; RNA binding

INTRODUCTION

Trypanosoma brucei is a flagellate protist of the order Kinetoplastida responsible for African sleeping sickness in humans and nagana in livestock. As one of the early diverged eukaryotes, it contains an array of remarkable biological properties that are not present in other eukaryotic cells. A prominent example is post-transcriptional uridine (U) insertion/deletion RNA editing of the mitochondrial (mt) transcripts. Only after being edited, the mRNAs become translatable, mainly encoding subunits of the respiratory complexes. The numerous insertions and deletions of Us are performed by the 20S editosome (also known as L-complex). The extensively characterized editosomes (for recent reviews, see Simpson et al. 2004; Lukeš et al. 2005; Stuart et al. 2005; Aphasizhev 2007) contain at least 20 proteins, and there are three types of 20S editosomes present in the organelle, each unique to one type of endonucleases (Panigrahi et al. 2006).

During editing, the sequence information is provided by small RNA molecules termed guide (g) RNAs. The role of mt RNA-binding proteins in this processing event is an anticipated feature that entails RNA/RNA hybridization. In addition to component proteins, proteins that are not stably associated with the editosome may be involved in the process and those proteins have been labeled as accessory factors (Stuart and Panigrahi 2002). There is a growing family of such proteins with multiple functions in mt RNA metabolism. The mt RNA-binding proteins (MRPs) are present in the MRP1/2 heterotetrameric complex that serves in the initial stage of RNA editing as a matchmaker by facilitating the hybridization of gRNA with its cognate pre-edited mRNA (Aphasizhev et al. 2003a; Schumacher et al. 2006). Moreover, a pleimorphic phenotype caused by the down-regulation of MRP1 and/or MRP2 indicated that apart from a role in editing, both proteins may also function in other aspects of RNA metabolism (Vondrušková et al. 2005). RBP16 is an RNA-binding protein that plays a role in RNA editing in vivo (Pelletier and Read 2003) and is able to stimulate insertion editing in vitro (Miller et al.

Reprint requests to: Julius Lukeš, Biology Centre, Institute of Parasitology, Branišovská 31, 37005 České Budějovice, Czech Republic; e-mail: jula@paru.cas.cz; fax: 00420-38-5310388.

Article published online ahead of print. Article and publication date are at http://www.rnajournal.org/cgi/doi/10.1261/rna.888808.

2006). Furthermore, it also influences the stability of never-edited RNAs as well as some of their edited counterparts (Pelletier and Read 2003) and is subject to arginine methylation that regulates its functions (Goulah et al. 2006). Another member of this rather heterogeneous family is REAP-1, a protein initially found to specifically bind to pre-edited mRNAs, thus being proposed to recruit pre-mRNAs to the editing complex (Madison-Antenucci et al. 1998). However, a recent study of REAP-1 knock-out cells implies a role in RNA stability rather than editing (Hans et al. 2007). Although RBP38 was first identified as a protein that binds gRNA–mRNA duplexes (Sbicego et al. 2003), its function seems to be in the initiation of kinetoplast DNA replication (Liu et al. 2006).

TbRGG1 is another protein ranked in the category of associated factors in RNA editing (Vanhamme et al. 1998). It contains the arginine-glycine-glycine (RGG) tripeptide, which is repeated within close proximity of each other and interspersed with aromatic residues, comprising a motif that is present in a number of RNA-binding proteins (Burd and Dreyfuss 1994). The positively charged arginines are thought to electrostatically interact with the negatively charged amino acids, thus facilitating RNA binding. TbRGG1 in *T. brucei* is an 85-kDa protein that is equally present in the procyclic and bloodstream stages. In glycerol gradients, TbRGG1 sedimented in 35–40 S fractions that also exhibited in vitro deletion activity, implying both association with a macromolecular complex and RNA editing (Vanhamme et al. 1998). The recombinant protein bound poly(U) with high affinity, a capacity suggestive of gRNA binding, since these molecules have 3′ oligo(U) tails (Blum and Simpson 1992). Interestingly, UV cross-linking of radiolabeled gRNAs to mitochondrial lysates yielded this protein (Leegwater et al. 1995). However, the evidence for function of TbRGG1 in RNA editing is ultimately circumstantial. Another mitochondrial RGG protein was initially identified by mass spectrometric analysis of the monoclonal antibody affinity purified editosome and the protein was called TbRGGm because of its homology with a *Trypanosoma cruzi* protein dubbed TcRGGm (Panigrahi et al. 2003a). Here, we present the resulting effect of the repression of TbRGG1 by RNA interference (RNAi) in *T. brucei*. Moreover, we report its RNase-sensitive association with a novel complex and identification of the components by mass spectrometry.

RESULTS

Silencing of TbRGG1 inhibits cell growth

An inhibition of growth is apparent 5 d after the induction of RNAi silencing of TbRGG1 (Fig. 1A). Thereafter, the induced cells grew approximately two times slower than the culture in which tetracycline induction was absent, and virtually stopped growing by day 12. The RNAi knock-

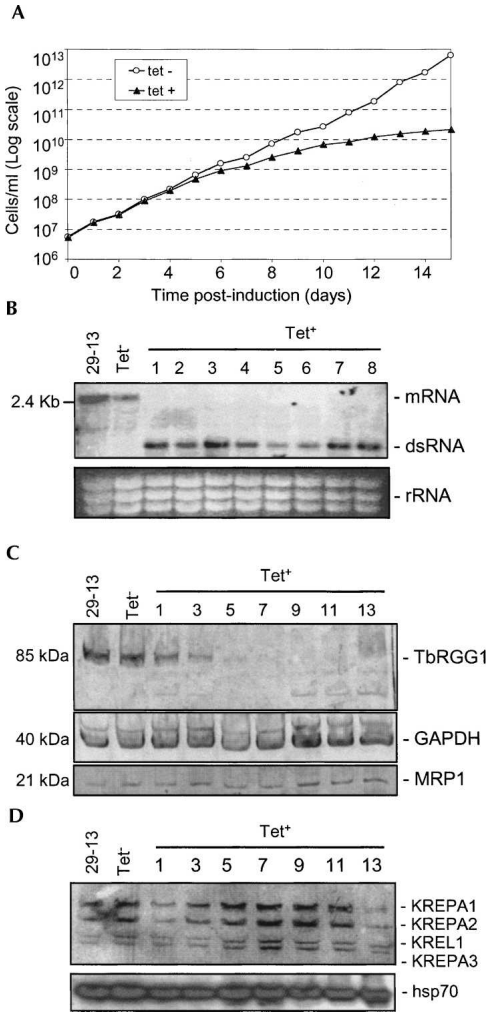

FIGURE 1. RNAi of TbRGG1 inhibits cell growth, and decreases protein and mRNA levels. (*A*) Growth effect. The numbers of noninduced cells (○) and those after induction by the addition of 1 μg/mL tetracycline (▲) were plotted logarithmically as the product of cell density and total dilution. Growth curves are one representative set from three independent experiments. (*B*) Effect on the TbRGG1 mRNA. Its levels were analyzed by blotting 10 μg of total RNA extracted from 29-13, noninduced (Tet−) and induced (Tet+) cells (days 1–8 post-induction of RNAi). The position of the targeted mRNA and the dsRNA synthesized following induction are indicated. As a loading control, the gel was stained with ethidium bromide to visualize rRNA bands. (*C*) Effect on the TbRGG1 protein. Its levels were analyzed by Western blot in extracts from 29-13, noninduced (Tet−) and induced (Tet+) cells lysed from day 1–13 every 48 h. Each lane was loaded with protein from ~5 × 10^6 cells and blots were immunodecorated using polyclonal antibody against TbRGG1, cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and mitochondrial RNA-binding protein 1 (MRP1). (*D*) 20 S editosome is unchanged. The editosome protein levels were analyzed by Western blots in mitochondrial extracts from 29-13 noninduced and induced cells lysed from day 1–13 every 48 h. Western blots were simultaneously probed with α-KREPA1, KREPA2, KREL1, and KREPA3 monoclonal antibodies (*top*), and polyclonal antibodies against the mitochondrial heat shock protein 70 (hsp-70) (*bottom*).

down cells showed no recovery even 2 wk after RNAi induction. The extent of TbRGG1 mRNA silencing, as well as the tightness of its inducible down-regulation, was determined by Northern analysis on RNA collected from parental 29-13, noninduced and 1–8 d post-induction cells (Fig. 1B). The target message was undetectable by 24 h after induction with tetracycline and remained so for at least 8 d. In addition, no leaky transcription from the p2T7-177 vector was evident, because dsRNA appeared only after RNAi induction (Fig. 1B). This observation is consistent with Western analysis, showing that the level of TbRGG1 protein significantly drops around day 5 postinduction (Fig. 1C), which corresponds nicely with the appearance of growth inhibition (Fig. 1A), and that its down-regulation persists over the 2 wk time course. In contrast, the signal from the GAPDH antibody, which was used as a loading control (Fig. 1C), persisted throughout the time course. Western analysis using monoclonal antibodies against the core editosome proteins KREPA1, KREPA2, KREL1, and KREPA3 (Fig. 1D) and the accessory complex protein MRP1 (Fig. 1C) showed no effect on abundance of these proteins consequent to TbRGG1 silencing.

It was originally reported that the ORF of TbRGG1 is present in steady-state levels on a dicistronic transcript, which contains another upstream ORF, as well as the expected processed monocistronic RNA (Vanhamme et al. 1998). Northern analysis was performed to confirm that the fully processed mRNA of the upstream ORF was not affected by the dsRNA targeting TbRGG1, and thus, that the observed growth inhibition was due only to TbRGG1 down-regulation (data not shown).

Reduction of edited RNAs upon TbRGG1 down-regulation

To determine whether TbRGG1 has a role in RNA editing, levels of several mt RNA species were compared between cultures growing for 4 d in the presence and absence of the RNAi induction agent tetracycline. This time point was chosen since it is just before observable growth inhibition (Fig. 1A), with the TbRGG1 protein being virtually eliminated (Fig. 1C). Quantitative real-time (q) PCR on cDNA from these cultures was performed with primers against several pre-edited, edited, and never-edited RNAs, as previously described (Carnes et al. 2005). Moreover, newly designed primers amplifying mt 9S and 12S rRNAs, as well as TbRGG1, were used. All qPCR reactions were performed in triplicate, including those for determination of the expression of β-tubulin and 18S rRNA, with an average and median standard deviation of the measured cycle threshold (C_t) values of 0.1 and 0.07, respectively (data not shown). The transcription levels of these housekeeping genes were unaltered by TbRGG1-interference, and thus served as references for calculation of the relative abundances of the other examined transcripts. The qPCR allowed quantification of the elimination of the TbRGG1 mRNA, which is reduced to about 20% of its normal level (Fig. 2).

The analysis of mt mRNAs revealed an overall decline of the levels of edited transcripts (Fig. 2). The edited cytochrome oxidase subunit 3 (co3) and NADH dehydrogenase subunit 7 (ND7) mRNAs decreased by about 50%, while the decline was less pronounced for ATPase subunit 6 (A6), maxicircle unknown reading frame 2 (MURF2), ribosomal protein S12 (RPS12), and co2 mRNAs (about 30%–40%), and only a subtle decrease was noted for edited cytochrome reductase subunit B (cyB). The decrease of edited molecules correlated with an increase of respective pre-edited mRNAs, especially for co3. There was virtually no change in the levels of never-edited ND4 and co1 mRNAs. The abundant mt 9S rRNA remained unaffected, while a slight decrease was apparent in 12S rRNA.

To investigate whether repression of TbRGG1 leads to any changes of the gRNA population, the same RNA used for qPCR assays was labeled with guanylyltransferase, an enzyme that specifically labels gRNAs, which can be subsequently visualized on a denaturing acrylamide gel (Fig. 3). A top band corresponding to a cytosolic RNA also labeled in this reaction (Aphasizhev et al. 2003b) was used as a

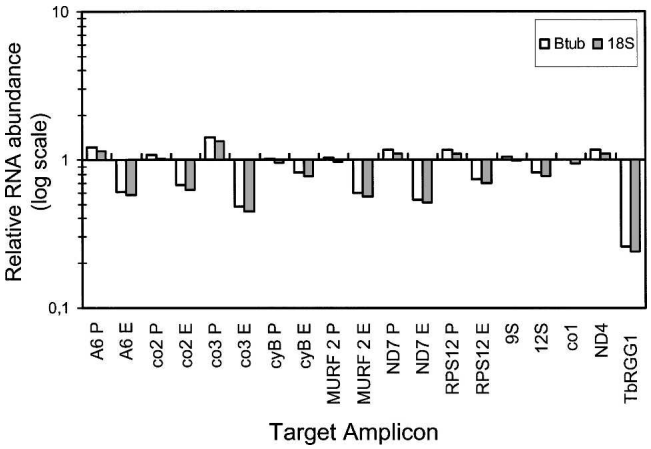

FIGURE 2. RNAi of TbRGG1 affects RNA editing. Real-time PCR analysis of pre-edited, edited, and never-edited mRNAs and ribosomal RNAs encoded in the mt genome. Analysis was performed in triplicate on cDNAs generated from cells grown for 4 d in the presence or absence of tetracycline. For each target amplicon, the relative change in RNA abundance due to induction of RNAi silencing of TbRGG1 was determined by using cytosolic transcripts of β-tubulin (white bar) and 18S rRNA (gray bar) as internal references, since their transcription was not affected. The relative abundance of each examined transcript upon synthesis of the TbRGG1 dsRNA was plotted on a logarithmic scale: 1.0 represents the wild-type level; levels above and below 1.0 mean an increase or decrease of a given RNA, respectively. The following pre-edited (P) and edited (E) mRNAs were assayed: ATPase subunit 6 (A6), cytochrome oxidase subunits 2 (co2), and 3 (co3), cytochrome reductase subunit b (cyB), maxicircle unknown reading frame 2 (MURF2), NADH dehydrogenase subunit 7 (ND7), and ribosomal protein S12 (RPS12). The following never-edited RNAs were assayed: 9S RNA, 12S RNA, co1, and ND4. The levels of the nuclear-encoded TbRGG1 mRNA were also tested (TbRGG1).

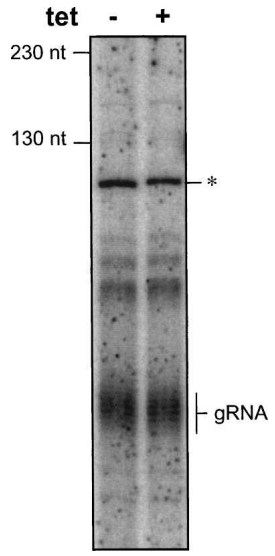

FIGURE 3. RNAi of TbRGG1 does not affect gRNAs. The total population of minicircle-encoded gRNAs was visualized in a high-resolution acrylamide-urea gel by labeling 2.5 μg of total RNA with guanylyltransferase and [γ-^{32}P]GTP. The *left* and *right* lane contains RNA from noninduced (Tet−) and induced (Tet+) TbRGG1 RNAi cells (4 d of induction), respectively. The gRNAs, which range in size mainly due to the variable lengths of their 3′-oligo(U) tails, are indicated on the *right*. The *top* band marked by the "*" is a cytosolic RNA that is concurrently labeled by the enzyme and is used as a loading control. The sizes of two in vitro-transcribed RNAs, which were end-labeled in separate reactions, are indicated on the *left*.

loading control between the samples from induced and noninduced cells. As judged by this assay, TbRGG1 repression does not significantly affect steady-state levels of gRNAs.

TbRGG1 is associated with a multiprotein complex

The sedimentation profile of TbRGG1 on a glycerol gradient as determined by Western analysis indicated that it is part of a large protein complex (Vanhamme et al. 1998). To explore this possibility, the TAP-tagged TbRGG1 protein was expressed in procyclic *T. brucei* cells under the control of a tetracycline-inducible promoter. Expression of the tagged protein was monitored on glycerol gradients using the PAP reagent, which specifically recognizes the Protein A domain of the tag. A strong band of 105 kDa showed the distribution of protein in fractions 3–13, with the bulk being present in fractions 5–9 (Fig. 4). Two additional bands are apparent below the major uppermost band, which may correspond to modification of TbRGG1 by proteolysis or other means, as observed and postulated in the original report (Vanhamme et al. 1998). The same gradient probed with anti-KREPA3 antibody showed the peak 20 S editosome signal in fraction 11 (Fig. 4). The sedimentation properties of the tagged TbRGG1 protein suggest its incorporation into a complex that overlaps with the 20 S editosome, although it sedimented at a somewhat lower "S" value than originally reported (Vanhamme et al. 1998), but consistently with earlier observation from our laboratory (Vondrušková et al. 2005).

The clarified lysate with the expressed TAP-tagged TbRGG1 was subjected to the tandem-affinity purification protocol. Western analysis of aliquots from the final four TAP eluates showed the presence of the tagged protein (Fig. 5A), and SYPRO Ruby staining of SDS-PAGE separated sample showed at least nine visible protein bands in the tagged TbRGG1 complex (Fig. 5B). The same fraction was also probed with a mix of monoclonal antibodies against the four core editosome proteins (KREPA1, KREPA2, KREL1, and KREPA3), and none of these reacted with the protein complex containing TbRGG1 (Fig. 5C). Thus, the tagged TbRGG1 complex does not associate with the editosomes.

Mass spectrometry analysis of the tagged TbRGG1 complex identified several proteins (Table 1; Supplemental Tables 1 and 2), including two proteins, Tb927.2.3800 and Tb927.7.2570, that have no known motifs but have 31% sequence identity and 48% similarity over 432 amino acids between them. These two proteins were identified in the sample with high peptide coverage (data not shown), thus, were selected for reciprocal TAP-tag analyses. Calmodulin elution fractions of Tb927.2.3800 and Tb927.7.2570 TAP-tag purifications were stained by SyproRuby (Fig. 6) and analyzed by LC-MS/MS, in which the TbRGG1 protein was also detected.

To assign the protein to this complex, the data from all three TAP-tag cell lines were compiled. Fourteen proteins that were identified with probability of ≥0.9 in all three TAP-tags, and at least by two unique peptide matches in one of them, were assigned as the component of the complex (Table 1). Seven other proteins that were identified using the above-mentioned conditions, but are known subunits of different mt complexes and/or are abundant mitochondrial and nonmitochondrial proteins, were operationally

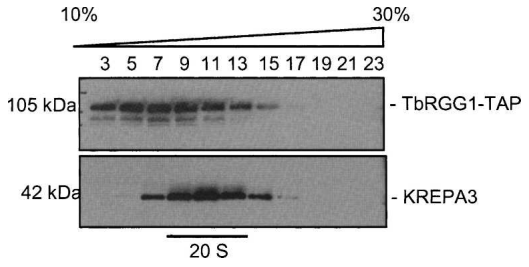

FIGURE 4. Sedimentation profile of the protein complex containing TbRGG1. The cleared lysate of hypotonically isolated mitochondria from cells containing the TAP-tagged TbRGG1 grown for 2 d in the presence of 500 ng/mL tetracycline were loaded onto glycerol gradients and fractionated as described in Materials and Methods. Fractions were immunodecorated with the PAP reagent, which binds the protein A domain of the TAP-tag (*top*), and the anti-KREPA3 (*bottom*) antibody as a marker of the 20 S fractions. The lower two bands visualized by the PAP reagent may correspond to modification of the TAP-tagged TbRGG1 protein by proteolysis or other means.

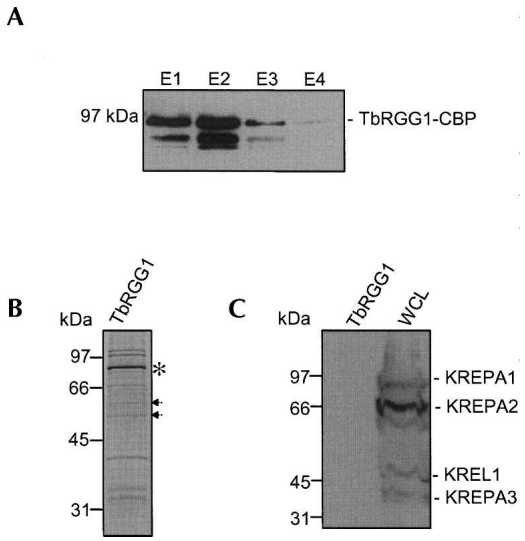

FIGURE 5. Identification of proteins associated with the TAP-tagged TbRGG1 protein. (*A*) Eluates E1–E4 from the TAP-tag purification using the TbRGG1 protein as bait were visualized by Western analysis using the anti-his antibody, recognizing the his-epitope present on the remaining part of the TAP-tag along with the calmodulin binding peptide (TbRGG1-CBP). The eluate most enriched for the TAP-tagged protein (E2) was subjected to LC-MS/MS. As in the glycerol gradient, two lower bands are apparent in the eluates enriched for the tagged protein, possibly products of the modification of the TbRGG1 portion of the fusion. (*B*) Eluate E2 was stained with Sypro Ruby. The predicted position of TbRGG1-CBP and the copurified proteins Tb927.2.3800 and Tb927.7.2570 are indicated on the *right* by the "*" and by the arrows, respectively. (*C*) The purified proteins from the E2 fraction were subjected to simultaneous immunoanalysis using the anti-KREPA1, KREPA2, KREL1, and KREPA3 monoclonal antibodies, using whole-cell lysates (WCL) from *T. brucei* as a positive control.

regarded as contaminants (Supplemental Table 1). We also list numerous proteins that were identified in one or two of the tagged complexes or identified only with one peptide match and were not considered as part of the complex (Supplemental Table 2).

Results from detailed sequence analysis of the component proteins with public databases are presented in Table 1. Some of the proteins in the complex have a high degree of conservation to other eukaryotic RNA helicases and RNA-binding proteins. Moreover, motifs involved in RNA–protein and protein–protein interactions, such as RRM, GRP, and ankyrin repeat were found within some of the polypeptide sequences. Interestingly, TbRGGm (Tb10.406.0050), a protein previously identified to be associated with an affinity purified editosome (Panigrahi et al. 2003a), was also among the RNA-binding motif containing proteins present in the complex. It is noteworthy that two pairs of closely related proteins were identified in the complex (Table 1).

Association of TbRGG1 with the multiprotein complex is RNA mediated

To test whether association of the TbRGG1 is mediated via RNA interactions, hypotonically isolated mitochondria from the 29-13 cell line were lysed in the presence or absence of RNase A, and the lysates were fractionated by sedimentation in glycerol gradients (Fig. 7A). As evident from Western analysis using the anti-TbRGG1 antibody, there is a pronounced shift in the localization of the TbRGG1 protein to the lighter fractions of the gradient upon RNase treatment, peaking in fractions 5 through 7 and absent after fraction 9. TbRGG1 in untreated samples peaks in fractions 7 through 11, and is detected throughout the gradient. The peak of fractionation of two subunits of the 20 S editosome, KREPA1 and KREPA2, was unaffected by RNase treatment (Fig. 7A).

In light of this result, the initial IgG affinity selection step was repeated for the TAP-tagged TbRGG1 in the presence and absence of RNase A. The eluates resulting from TEV cleavage, which releases protein(s) bound to the IgG matrix by the TAP-tag, were resolved by glycerol gradient centrifugation. A shift in the sedimentation of TAP-tagged TbRGG1 to the lighter fractions is apparent when the ribonuclease is present during the first purification step (Fig. 7B). SyproRuby staining of the calmodulin eluates directly separated on SDS-PAGE gel reveals that the abundant bands corresponding in size to the Tb927.2.3800 and Tb927.7.2570 proteins disappear in the presence of RNase A (Fig. 7C). Furthermore, only TbRGG1 peptides were detected in these eluates by mass spectroscopy analysis. Reciprocally, TbRGG1 was not detected by this method in Tb927.2.3800 TAP eluates purified in the presence of RNase A, while other component proteins of the complex were present, as compared with parallel untreated purifications (data not shown). We propose that this novel multiprotein complex is involved in mt RNA metabolism based on the observations that TbRGG1, a protein with such a role, interacts with it in an RNA-mediated manner, and that it contains several proteins with RNA-binding and processing motifs.

DISCUSSION

Since its serendipitous discovery almost 10 yr ago (Vanhamme et al. 1998), TbRGG1 has remained among the least-characterized proteins putatively associated with RNA editing, while the functions of other RNA editing accessory factors have been elucidated, at least to some extent. It was proposed that TbRGG1 may have a role in RNA editing and that it is part of a macromolecular complex based on its reported biochemical properties (Vanhamme et al. 1998; Simpson et al. 2004; Lukeš et al. 2005; Stuart et al. 2005). Here, we test both of these hypotheses in vivo using reverse genetics and TAP-purification approaches that have been established in *T. brucei* subsequent to the discovery of this protein.

Using an RNAi-based approach, we have generated cells deficient for TbRGG1 and showed that editing of all analyzed mRNAs is down-regulated, though to varying degrees, whereas the assayed never-edited mRNAs remain at their wild-type levels. A similar phenotype has been

TABLE 1. Mitochondrial proteins associated with the TAP-tagged TbRGG1 protein, Tb927.2.3800, and Tb927.7.2570 protein in procyclic *T. brucei*

Protein name	GeneDB	TbRGG1	Tb927.2.3800	Tb927.2.2570	Homology (*E*-value)	Motif/domain (*E*-value)
Tb927.6.2230^{tag}	TbRGG1	√	√	√	*Caenorhabditis* hypothetical protein (4e-13)	—
Tb927.7.2570^{tag}	HP*	√	√	√	Tb927.2.3800[1]	—
Tb927.2.3800^{tag}	HP*	√	√	√	Tb927.7.2570[1]	—
Tb927.5.3010	HP	√	√	√	*Babesia* hypothetical protein (9e-09); bacterial putative RNA-binding protein (6e-06)	Ribosomal S2 protein signature
Tb927.4.1500	HP	√	√	√	*Oryza* ATP-dependent RNA helicase (1e-121)	dsrm (9e-07); Helicase C (2e-13)
Tb11.02.5390	HP	√	√	√	—	Ankyrin repeat (0.83)
Tb11.01.8620	HP	√	√	1	—	—
Tb10.406.0050	RBP$^{\#}$	√	√	√	*Caenorhabditis* RNA helicase (1e-09)	RRM (6.3e-11)
Tb927.8.8170	HP	√	√	1	Tb927.4.4160[2]	—
Tb927.4.4160	HP	√	1	√	Tb927.8.8170[2]	—
Tb927.3.1820	HP	√	√	√	—	GRP (0.028)
Tb11.01.7290	Nudix hydr*	√	√	1	—	NUDIX (4e-05)
Tb927.3.4920	HP	1	√	√	*Arabidopsis* calcium binding mt protein (9e-06)	LETM1 (4e-08)
Tb927.6.2140	HP	1	√	1	Bacterial hydratase (6e-05)	2-keto-4 pentenoate hydratase (5e-07)

(√) Protein was identified with more than two unique tryptic peptides; (1) protein was identified with 1 unique tryptic peptide; (*) proteins also known as LtAP1-3 identified in *L. tarentolae* MRP1/2 complex (3); (superscript "tag") tagged proteins in this study; (superscript "#") protein also known as TbRGGm (20); (superscript "1") proteins have 31% sequence identity and 48% similarity over 432 amino acids between them; and (superscript "2") proteins have 77% sequence identity and 85% similarity over 904 amino acids between them.

observed when subunits of the 20 S editosome are silenced, which have a direct role in RNA editing (Carnes et al. 2005; Trotter et al. 2005; Salavati et al. 2006). This result is noteworthy when compared with studies in which the expression of other RNA editing accessory factors is disrupted. In the procyclic stages, RNAi knock-down of either of the two subunits of the MRP1/2 complex decreased the levels of a subset of edited and never-edited mRNAs (Vondrušková et al. 2005). Gene knock-out of *MRP1* in the bloodstages had a comparable effect (Lambert et al. 1999), while silencing of RBP16 caused a reduction of edited cyB and never-edited RNAs (Pelletier and Read 2003). Such a pleimorphic effect on mt transcripts is not observed when TbRGG1 is silenced.

Because of its in vitro poly(U)–RNA binding activity, the TbRGG1 protein was suggested to have an alternate role in the synthesis and/or processing of those mt RNAs (Leegwater et al. 1995; Vanhamme et al. 1998) that contain the post-transcriptionally added 3′-oligo(U) extension (Aphasizhev 2007). This possibility was also tested by assaying the levels of the mitoribosomal RNAs in parallel with the other mRNAs by qPCR. In the induced cells, the levels of the 9S and 12S rRNAs remain unchanged and slightly reduced, respectively, implying that TbRGG1 does not have the proposed role in mt rRNA synthesis.

Another prominent class of mt RNAs with added 3′-oligo(U) tails are the gRNAs, which supply the essential information for proper U-insertion/deletion into the cognate mRNA (Blum and Simpson 1992). Because of its aforementioned affinity for poly(U), it was suggested that TbRGG1 interacts directly with gRNAs, perhaps functioning in their stabilization or maturation. As judged by the

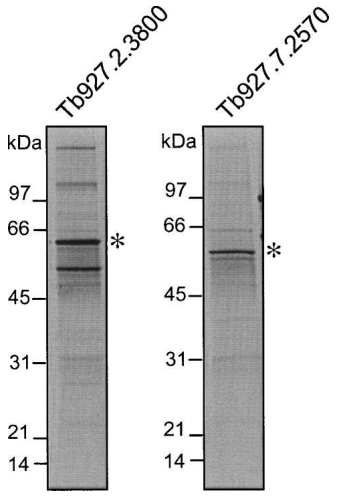

FIGURE 6. Sypro Ruby staining of purified Tb927.2.3800 (*left*) and Tb927.7.2570 (*right*) complexes. The predicted position of the tagged proteins (Tb927.2.3800-CBP or Tb927.7.2570-CBP) are indicated on the *right*.

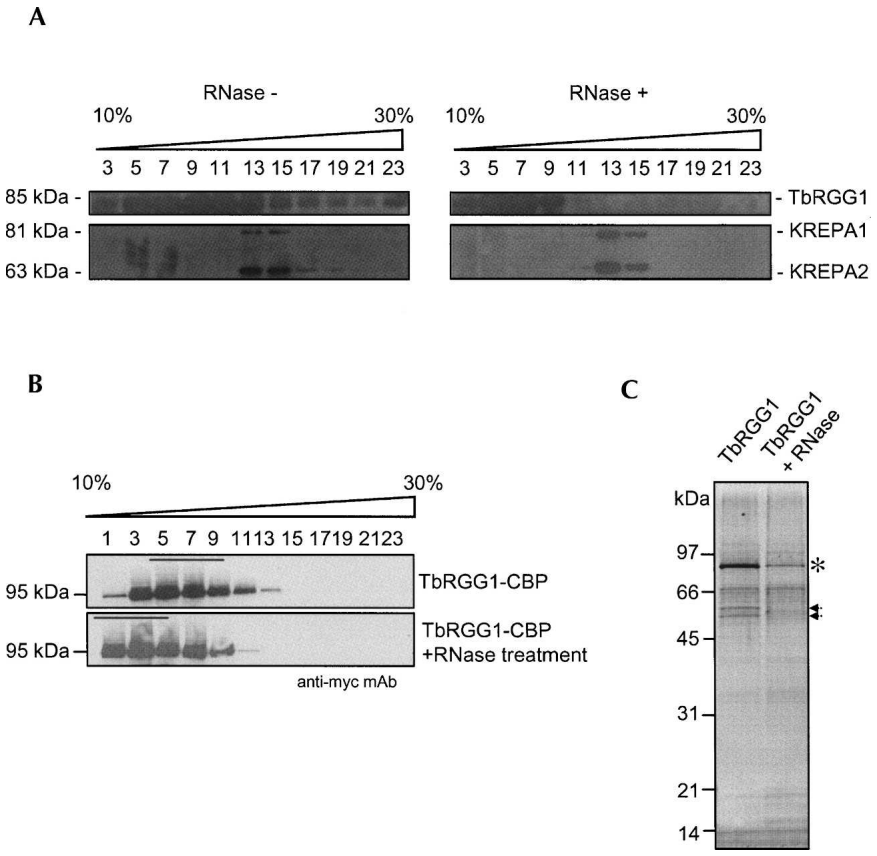

FIGURE 7. TbRGG1 association with the MRB complex 1 appears to be RNase sensitive. (*A*) Hypotonically isolated mitochondria from the 29-13 cell line were lysed in the absence (*left*, RNase-) or presence (*right*, RNase+) of 0.1 mg/mL RNase A. The latter lysates were loaded onto glycerol gradients containing the same concentration of the enzyme. Western analysis was done on resulting fractions with the anti-TbRGG1 antibodies, as well as the anti-KREPA1 and anti-KREPA2 antibodies as markers of the 20 S fractions. (*B*) IgG purification of the TAP-tagged TbRGG1 in the presence and absence of RNase A. The eluates resulting from TEV cleavage, which releases protein(s) bound to the IgG matrix by the TAP-tag, were resolved by glycerol gradient centrifugation. A shift in the sedimentation of TAP-tagged TbRGG1 to the lighter fractions is apparent when the ribonuclease is present during the first purification step. The peak fraction designated by a black line was pooled and further subjected to the second affinity purification step. (*C*) SyproRuby staining of the calmodulin eluates purified from pooled glycerol gradient fractions separated on 10.5%–14% SDS-PAGE gel shows that the abundant bands corresponding in size to the Tb927.2.3800 and Tb927.7.2570 proteins disappear in the presence of RNase A, which are indicated by arrows. TbRGG1-CBP is designated with "*."

guanylyltransferase labeling assay, no significant changes are observed to the steady-state levels of gRNAs or their processing upon TbRGG1 silencing.

One plausible interpretation of these results is that TbRGG1 has a role in RNA editing, since the effect of its silencing is limited to a decrease in edited mRNAs as compared with the other assayed transcripts. However, it most likely does not have a direct role in the process, since the observed down-regulation occurs without a corresponding accumulation of pre-edited RNAs, as observed when multiround editing is disrupted by silencing of the components of the 20 S editosome (Carnes et al. 2005; Trotter et al. 2005; Salavati et al. 2006). An indirect role of TbRGG1 in this process may be via interaction with the gRNAs requisite for decrypting the appropriate mRNAs. Although the guanylyltransferase labeling of these molecules has shown that their stability and processing remain intact upon repression of TbRGG1, the possibility that the protein binds the gRNAs in order to facilitate their utilization, a role proposed for RBP16 (Pelletier and Read 2003), remains open. However, the reduction of edited co2 mRNA in the TbRGG1 knockdowns is quite telling, since its gRNA is located within its 3′-UTR, thus not requiring the independent *trans*-acting gRNA molecules (Kim et al. 1994). TbRGG1 binding to gRNAs, which are heterogeneous in sequence, would most likely involve conserved features such as the 3′ oligo(U) tail, which are absent in the co2 *cis*-gRNA, as implied by its incapacity to act in *trans* (Golden and Hajduk 2005). Thus, based on the observation that co2-edited RNAs are affected by TbRGG1 depletion, while gRNA levels are not, we conclude that the protein does not act through these small RNAs. An explanation that is consistent with the in vivo data is that TbRGG1 may be involved in the stabilization of edited RNAs. Alternately, it may have a role in editing efficiency, since depletion of a protein with such a function would also result in a decrease of edited RNAs.

It was postulated that TbRGG1 is associated with a protein complex performing key editing activities, which is now the well-known 20 S editosome or L-complex. Yet, detailed analyses of the composition of the editosome(s) in *T. brucei* failed to identify TbRGG1 in them (Aphasizhev et al. 2003b; Panigrahi et al. 2003a,b, 2006). Indeed, RNAi silencing of TbRGG1 did affect neither the editosome nor the MRP1/2 complex, since subunits incorporated into either of these complexes remained at wild-type levels, an indication of their integrity (Wang et al. 2003; Vondrušková et al. 2005). To characterize the composition of the complex predicted to interact with TbRGG1, overexpression of the TAP-tagged protein was performed with the intention of identifying any copurified subunits by mass spectrometry. The sedimentation properties in glycerol gradients of tagged TbRGG1 suggest incorporation into a complex, although in lower density fractions than initially reported (Vanhamme et al. 1998). The discrepancy in the described sedimentation

properties of TbRGG1 may be due to technical differences in gradient preparation in the two studies, as the localization of in vitro editing activity, found in the same fractions as TbRGG1 originally (Vanhamme et al. 1998), was at a higher density than is typically observed (Stuart et al. 2005).

The association of TbRGG1 with proteins that may represent a separate complex was demonstrated by the coisolation of a novel set of proteins with TAP-purified TbRGG1, as detected by mass spectrometry. The association was further validated by tagging two other component proteins of the complex. Based on the TAP-tag analyses, we assigned 14 proteins to this novel putative complex, which include a Nudix hydrolase, RNA helicase, TbRGGm, and hypothetical proteins with and without identifiable motifs. A probable TbRGG1 interaction with this complex via RNA was also revealed. This association is likely to be biologically relevant, as it is reproducibly detected in TbRGG1, Tb927.2.3800, and Tb927.7.2570 TAP purifications, without consistently revealing other RNA-binding proteins such as the MRPs, RBP16, REAP1, subunits of the 20 S editosome or ribosomal proteins that may potentially associate with the TAP-tagged proteins nonspecifically due to their affinity for nucleic acids. However, because the complex interacts in an RNase-sensitive manner with TbRGG1 and contains multiple proteins with RNA-binding domains suggestive of roles in RNA processing, we provisionally name it the putative mitochondrial RNA-binding complex 1 (put-MRB complex 1).

Interestingly, three of the proteins identified to be components of the MRB complex 1 were previously encountered in substoichiometric amounts in TAP-purifications of the MRP1/2 complex from *Leishmania tarentolae*: Tb927.2.3800 (LtAP-1), Tb927.7.2570 (LtAP-2), and Nudix hydrolase (LtAP-3) (Aphasizhev et al. 2003a). In contrast, TAP or monoclonal antibody affinity purification of the MRP1/2 complex in *T. brucei* did not copurify these proteins (Panigrahi et al. 2008; Zíková et al. 2008), and we have not identified the MRP1 or MRP2 proteins as part of put-MRP complex 1. Another purification of the putative complex, using a slightly different approach and yielding an overlapping subunit composition also did not contain any MRP peptides (Panigrahi et al. 2008). This discrepancy may be due to RNA-based interactions between these proteins; technical differences in the manner the purifications were performed, such as the amount of cells used, or it simply reflects the different types of associations that take place in the two species.

In conclusion, the 14 proteins identified using tagged TbRGG1, Tb927.2.3800, and Tb927.7.2570 seem to be genuine components of a novel ribonucleoprotein complex or a collection of complexes and/or monomers that associate via RNA linkers. We have termed it here the put-MRB complex 1, since it has features that suggest a function in RNA processing activities in *T. brucei* procyclics, and perhaps an indirect role in RNA editing. This working hypothesis is supported by the effect of RNAi silencing of TbRGG1 on edited RNAs as well as by its association with proteins containing motifs that mediate RNA metabolism and/or protein–protein interactions. In addition, we provide evidence that the association of TbRGG1 with this complex may be mediated through RNA. We are in the process of extensive characterization of the function and composition of this enigmatic protein complex.

MATERIALS AND METHODS

Plasmid construction, cell culture, transfection, RNAi induction, and growth curves

A 543-nt long N-terminal region of the TbRGG1 gene overlapping the RGG motif was PCR amplified using primers RG-F (5′-GGATCCACTACCGAGATCAGCGCAAC) and RG-R (5′-AAGCTTGCATCCATTCTGTAGCCTG) (added BamHI and HindIII restriction sites underlined) from genomic DNA of the *T. brucei* strain 29-13. The amplified fragment was cloned into the p2T7-177 vector, which was electroporated into procyclic *T. brucei* strain 29-13 and selected following a protocol described elsewhere (Vondrušková et al. 2005). The synthesis of dsRNA was induced by the addition of 1 μg/mL tetracycline and the cell density was measured every 24 h. Cell growth rate was determined using the Beckman Z2 Coulter counter over a period of 15 d after the induction of RNAi.

To create a construct for the inducible expression of C-terminally TAP-tagged proteins, the full-length TbRGG1 gene was amplified with primers RG-TAP-Fw (5′-GGGCACAAGCTTATGGTGTGTAGC) and RG-TAP-Rv (5′-GGCGGAGGATCCGCTGTCTTCCAGCC) (added HindIII and BamHI restriction sites underlined) and inserted into the pLew79-MHTAP plasmid (Panigrahi et al. 2003b; Jensen et al. 2006). Similarly, Tb927.2.3800 and Tb927.7.2570 genes were PCR amplified and cloned into pLew79-MHTAP plasmid. The Tb927.2.3800 ORF was PCR amplified from genomic DNA of the 29-13 strain using primers Zah1-TAP-Fw2 (5′-ATTTCATGCTGCGCGCGCGCCTG) and Zah1-TAP-Rv (5′-AAAGGATCCGTATGCCGAAACGGCAGTC). This PCR product was used as a template for PCR with primers Zah1-TAP-Fw (5′-CTGTACTATATTGAAGCTTATGCTGCG) and the aforementioned Zah1-TAP-Rv containing the appropriate HindIII and BamHI restriction sites for cloning, as indicated with underlines. The Tb927.7.2570 ORF was PCR amplified from genomic DNA with primers Zah2-TAP-Fw (5′-TTGACCTCGAGATGCTTCGC) and Zah2-TAP-Rv (5′-GAGATCCAGATCTCAACTTCGC) (XhoI and BglII sites are underlined). The NotI-linearized constructs were introduced into the 29-13 cells and phleomycin-resistant clones were selected.

Northern blot analyses

Total RNA was isolated using Trizol (Sigma) from cells collected at regular intervals and 10 μg were loaded on a 1% formaldehyde agarose gel, blotted, and cross-linked following standard protocols. After prehybridization in Church–Gilbert solution for 1 h at 65°C, hybridization with a radiolabeled probe was performed overnight in the same solution and at the same temperature. Membranes were washed with 2x SSC + 0.1% SDS for 15 min at room temperature, followed by two washes with 0.2x SSC + 0.1%

SDS for 20 min each at 55°C. The radioactive signals were visualized using the PhosphorImager Storm 860.

Western blot analyses

Lysates in SDS-PAGE loading buffer from an equivalent of 5 × 10^6 cells/lane were separated either on 10% or 4%–15% SDS-PAGE gradient gels. Proteins were transferred onto nitrocellulose membrane by electroblotting, blocked overnight with 5% (w/v) nonfat milk in the same solution, and probed with either the anti-TbRGG1 antiserum (provided by L. Vanhamme) or the polyclonal rabbit antisera against *T. brucei* MRP1 (Vondrušková et al. 2005), GAPDH (provided by P.A.M. Michels) and hsp70, all at dilution 1:5000. Monoclonal antibodies against KREPA1 (TbMP81), KREPA2 (TbMP63), KREL1, and KREPA3 (TbMP42) were used as described elsewhere (Panigrahi et al. 2001b) on lysates from hypotonically isolated mitochondria.

Small-scale isolation of mitochondria

A pellet of 10^8 cells was resuspended in 1.5 mL of cold hypertonic NET buffer (100 mM NaCl, 100 mM EDTA at pH 8.0, 10 mM Tris-HCl at pH 7.9), incubated on ice for 10 min, then spun down, and the supernatant was discarded. The pellet was resuspended in cold hypotonic DTE buffer (1 mM Tris-HCl at pH 7.9, 1 mM EDTA at pH 8.0), which disrupts the plasma membrane, and the suspension was passed through a 25-gauge needle into 170 μL of 60% sucrose. The mitochondrial vesicles were spun down and the cytosolic content in the supernatant was discarded. The pelleted mitochondria were resuspended in 500 μL of STM buffer (250 mM sucrose, 20 mM Tris-HCl at pH 7.9, 2 mM $MgCl_2$) supplemented with 3 mM $MgCl_2$ and 5 U DNase I and left on ice for 1 h. Half a mL of STE was added to stop the reaction and the mitochondria were pelleted and washed two more times in 500 μL of STE (250 mM sucrose, 20 mM Tris-HCl at pH 7.9, 2 mM EDTA at pH 8.0) in the same manner. The mitochondrial pellets were stored at −80°C until use.

Quantitative real-time PCR

Ten micrograms of total RNA isolated from noninduced TbRGG1 RNAi cells and cells following 4 d of RNAi induction were treated with the Turbo DNA free kit (Ambion) following the manufacturer's protocol. After the DNase step, 1 μg of RNA was run on a formaldehyde gel to check the integrity of the treated RNA. To prepare templates for quantitative real time (q) PCR, cDNA was synthesized from 4.5 μg of RNA with the SuperScript III reverse transcriptase (Invitrogen) using random hexamers in a volume of 20 μL. A parallel reverse transcription reaction was also performed with the same amount of RNA and omitting the reverse transcriptase as a control for genomic DNA contamination. Both cDNA reactions were diluted 1:10 in H_2O prior to qPCR.

The qPCR reactions were performed using the Rotor-Gene 3000 Real-Time DNA Detection System thermocycler, and data, including PCR efficiencies, were acquired using the Rotor-Gene v6.1 software (Corbett Life Science). The primer pairs used to detect mt transcripts were described previously (Carnes et al. 2005), as are those amplifying 18S rRNA and β-tubulin cDNAs, which were used as reference genes. Primer pairs to detect the 9S and 12S mitochondrial rRNAs and TbRGG1 transcripts were designed for this study and are as follows:

9S Forward (5′-AATGCTATTAGATGGGTGTGGAA-3′);
9S Reverse (5′-GCTGGCATCCATTTCTGACT-3′);
12S Forward (5′-GGGCAAGTCCTACTCTCCTTT-3′);
12S Reverse (5′-TGCCCCAATCAAACATACAA-3′);
TbRGG1 Forward (5′-TTTGACGACCCAAGCACTATGTT-3′); and
TbRGG1 Reverse (5′-GCCTCCCAGCGGTCCTAT-3′).

The TbRGG1 primer pair is not complementary to the dsRNA region targeting the transcript. For each primer, 4 μL of a 1.5 μM stock was added to a 20-μL reaction, in addition to 10 μL of the Power SYBR Green PCR Master Mix (Applied Biosystems) and 2 μL of the cDNA. In the case of reference gene qPCR reactions, the cDNA was additionally diluted 1:50. The qPCR program is as follows: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 sec at 95°C and 60 sec at 60°C, in which fluorescence were detected at the end of the latter step. The correctness of the amplicon was verified both by a final dissociation step and electrophoresis of the qPCR product on a 2% agarose gel. The relative abundance of RNAs between the two samples was determined by the Pfaffl method (Pfaffl 2001).

Guanylyltransferase labeling

To visualize gRNAs, 2.5 μg of total RNA from the noninduced and induced (4 d) cells was DNase treated as described above and labeled using guanylyltransferase. A 15-μL reaction contained 1.5 μL of the 10x Capping Reaction Buffer (500 mM Tris at pH 8.0, 60 mM KCl, 12.5 mM DTT, 12.5 $MgCl_2$, 0.5 mg/mL BSA, 45 μCi of [α-^{32}P]GTP, 40 U RNaseOUT Ribonuclease Inhibitor (Invitrogen), and 5 U of guanylyltransferase (Ambion). The reaction was incubated for 1 h at 37°C and stopped by the addition of 0.1% SDS and 10 mM EDTA. The reaction products were phenolchloroform extracted, ethanol precipitated, and dissolved in 8 μL of 60% formamide. They were incubated at 80°C for 2 min, and 4 μL were loaded on a high-resolution denaturing 12% acrylamide-8 M urea gel.

Glycerol gradient sedimentation

For hypotonic purification of the mitochondria, the pellet from 10^{10} cells was washed in 35 mL of the SBG buffer (100 mM NaCl; 20 mM glucose; 20 mM $NaHPO_4$ at pH 7.9) and then dounce homogenized in 35 mL of the DTE solution. Next, 5.85 mL of 60% sucrose was added and the hypotonically isolated mt vesicles were pelleted and resuspended in 6.8 mL of the STM buffer, which was supplemented with 21 μL each of 1 M $MgCl_2$ and 0.1 M $CaCl_2$, and 61.2 μg of DNase. The DNase treatment was stopped with equal volume of the STE buffer. The enriched mitochondria were then lysed in 1 mL of the lysis buffer (10 mM Tris at pH 7.2, 10 mM $MgCl_2$, 100 mM KCl, 1 mM DTT, 1 μg/mL pepstatin, 2 μg/mL leupeptin, 1 mM pefabloc) containing 1% Triton X-100, for 15 min at 4°C. The lysate was cleared by centrifugation and loaded onto a 10%–30% glycerol gradient containing the same concentration of DTT and protease inhibitors as the lysis buffer. The gradient was spun at 38,000 rpm in a SW 40 Ti rotor (Beckman) for 5 h at 4°C, and 500-μL fractions were subsequently collected from the top. In experiments testing RNase sensitivity of TbRGG1 interaction with macromolecular complexes, 0.1 mg/mL of RNase A was introduced into the lysis buffer and glycerol gradient, which was spun using the previously mentioned rotor at

38,000 rpm for 12 h at 4°C or 0.1 mg/mL of RNase A was added during IgG affinity step, and TEV eluates were loaded on glycerol gradients and spun at 38,000 rpm for 5 h at 4°C.

TAP-purification of TbRGG1

Either the whole cell pellet or hypotonically isolated mitochondria (scaled up from the protocol described above) from 4×10^{10} cells containing the TbRGG1 gene with a tandem affinity purification (TAP) tag, whose expression was induced by 500 ng/mL tetracycline, were lysed on ice for 20 min in 20 mL of the IPP150 buffer (10 mM Tris-HCl at pH 8.0, 150 mM NaCl, 0.1% NP40) containing 1% Triton X-100 and two tablets of Complete EDTA-free protease inhibitors (Roche). After centrifugation at 9000*g* for 30 min, the cleared supernatant was incubated with 200 μL of the IgG Sepharose Fast Flow beads (Pharmacia) for 2 h at 4°C. After draining the supernatant and wash in IPP150, beads were incubated with 100 U of TEV protease (Invitrogen) in 1 mL of the TEVCB buffer (10 mM Tris-HCl at pH 8.0, 150 mM NaCl, 0.1% NP40, 0.5 mM EDTA, 1 mM DTT) for 2 h at 16°C. The TEV eluate was combined with three times the volume of calmodulin resin (Stratagene) in the CBB buffer (10 mM Tris-Hcl at pH 8.0, 150 mM NaCl, 0.1% NP40, 10 mM β-mercaptoethanol [ME], 1 mM Mg acetate, 1 mM imidazole, 2 mM $CaCl_2$), which was incubated for 1 h at 4°C. The tagged complexes were eluted with $4 \times$ 250 μL of the CEB buffer (10 mM Tris-Hcl at pH 8.0, 150 mM NaCl, 0.1% NP40, 10 mM ME, 1 mM Mg acetate, 1 mM imidazole, 2 mM EGTA). The same protocol was used to purify proteins associated with TAP-tagged Tb927.2.3800 and Tb927.7.2570 proteins in corresponding cell lines induced with tetracycline. TAP purification Tb927.2.3800 was performed in parallel in the presence and absence of 0.1 mg/mL RNase A during the initial incubation of the cleared lysate with the IgG Sepharose beads. The proteins from isolated complexes were separated in 10.5%–14% SDS-PAGE gel and visualized by SYPRO Ruby (Invitrogen) staining.

Mass spectrometry analysis

Proteins were analyzed by mass spectrometry as described (Panigrahi et al. 2001a). Briefly, proteins in 100 μL of the TAP eluate were precipitated with 600 μL of acetone, resuspended in 8 M urea/1 mM DTT, and denatured at 50°C for 1 h The sample was diluted 1:7 with 50 mM NH_4-HCO_3 and treated with 100 ng of mass spectrometry grade trypsin (Promega) at 37°C overnight. The mixture was dried in a speedvac and dissolved in 5% acetonitrile/0.4% acetic acid. The resulting peptides were purified using a C18 ZipTip (Millipore) following the manufacturer's protocol. The peptides were analyzed using a LTQ Linear Ion Trap Mass Spectrometer. The CID spectra were compared with the *T. brucei* protein database downloaded from GeneDB using TurboSequest software, and protein matches were determined using PeptideProphet and ProteinProphet programs (Keller et al. 2002; Nesvizhskii et al. 2003).

Sequence analysis

The protein sequences were compared with the NCBI nr database using PSI-BLAST and motif, and domain searches were carried out by CDD, PFAM, and Prosite searches.

SUPPLEMENTAL DATA

Supplemental material can be found at http://www.rnajournal.org.

ACKNOWLEDGMENTS

We thank Zdeňka Číčová (Biology Centre) for help with some experiments and Yuko Ogata (Seattle Biomedical Research Institute) for help with mass spectrometry analysis. Paul A.M. Michels (De Duve Institute of Cellular Pathology, Brussels) and Luc Vanhamme (Free University of Brussels, Gosselies) kindly provided antibodies. This work was supported by the Grant Agency of the Czech Republic 204/06/1558, Grant Agency of the Czech Academy of Sciences A500960705, and the Ministry of Education of the Czech Republic (LC07032, 2B06129, and 6007665801) to J.L., the Ph.D. student grant 524/03/H133 to H.H., and National Institutes of Health Grants 5R03TW6445 (to K.D.S. and J.L.) and AI14102 (to K.S.).

Received October 23, 2007; accepted February 15, 2008.

REFERENCES

Aphasizhev, R. 2007. RNA editing. *Mol. Biol.* **41:** 227–239.

Aphasizhev, R., Aphasizheva, I., Nelson, R.E., and Simpson, L. 2003a. A 100-kDa complex of two RNA-binding proteins from mitochondria of *Leishmania tarentolae* catalyzes RNA annealing and interacts with several RNA editing components. *RNA* **9:** 62–76.

Aphasizhev, R., Aphasizheva, I., Nelson, R.E., Gao, G., Simpson, A.M., Kang, X., Falick, A.M., Sbicego, S., and Simpson, L. 2003b. Isolation of a U-insertion/deletion editing complex from *Leishmania tarentolae* mitochondria. *EMBO J.* **22:** 913–924.

Blum, B. and Simpson, L. 1992. Formation of guide RNA messenger-RNA chimeric molecules in vitro, the initial step of RNA editing, is dependent on an anchor sequence. *Proc. Natl. Acad. Sci.* **89:** 11944–11948.

Burd, C.G. and Dreyfuss, G. 1994. Conserved structures and diversity of functions of RNA-binding proteins. *Science* **265:** 615–621.

Carnes, J., Trotter, J.R., Ernst, N.L., Steinberg, A.G., and Stuart, K. 2005. An essential RNase III insertion editing endonuclease in *Trypanosoma brucei*. *Proc. Natl. Acad. Sci.* **102:** 16614–16619.

Golden, D.E. and Hajduk, S.L. 2005. The 3′-untranslated region of cytochrome oxidase II mRNA functions in RNA editing of African trypanosomes exclusively as a *cis* guide RNA. *RNA* **11:** 29–37.

Goulah, C.C., Pelletier, M., and Read, L.K. 2006. Arginine methylation regulates mitochondrial gene expression in *Trypanosoma brucei* through multiple effector proteins. *RNA* **12:** 1545–1555.

Hans, J., Hajduk, S.L., and Madison-Antenucci, S. 2007. RNA-editing-associated protein 1 null mutant reveals link to mitochondrial RNA stability. *RNA* **13:** 881–889.

Jensen, B.C., Kifer, C.T., Brekken, D.L., Randall, A.C., Wang, Q., Drees, B.L., and Parsons, M. 2006. Characterization of protein kinase CK2 from *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* **151:** 28–40.

Keller, A., Nesvizhskii, A.I., Kolker, E., and Aebersold, R. 2002. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal. Chem.* **74:** 5383–5392.

Kim, K.S., Teixeira, S.M., Kirchhoff, L.W., and Donelson, J.E. 1994. Transcription and editing of cytochrome oxidase II RNAs in *Trypanosoma cruzi*. *J. Biol. Chem.* **269:** 1206–1211.

Lambert, L., Muller, U.F., Souza, A.E., and Göringer, H.U. 1999. The involvement of gRNA-binding protein gBP21 in RNA editing-an

in vitro and in vivo analysis. *Nucleic Acids Res.* **27:** 1429–1436.

Leegwater, P., Speijer, D., and Benne, R. 1995. Identification by UV cross-linking of oligo(U)-binding proteins in mitochondria of the insect trypanosomatid *Crithidia fasciculata*. *Eur. J. Biochem.* **227:** 780–786.

Liu, B., Molina, H., Kalume, D., Pandey, A., Griffith, J.D., and Englund, P.T. 2006. Role of p38 in replication of *Trypanosoma brucei* kinetoplast DNA. *Mol. Cell. Biol.* **26:** 5382–5393.

Lukeš, J., Hashimi, H., and Zíková, A. 2005. Unexplained complexity of the mitochondrial genome and transcriptome in kinetoplastid flagellates. *Curr. Genet.* **48:** 277–299.

Madison-Antenucci, S., Sabatini, R.S., Pollard, V.W., and Hajduk, S.L. 1998. Kinetoplastid RNA-editing-associated protein 1 (REAP-1): A novel editing complex protein with repetitive domains. *EMBO J.* **17:** 6368–6376.

Miller, M.M., Halbig, K., Cruz-Reyes, J., and Read, L.K. 2006. RBP16 stimulates trypanosome RNA editing in vitro at an early step in the editing reaction. *RNA* **12:** 1292–1303.

Nesvizhskii, A.I., Keller, A., Kolker, E., and Aebersold, A. 2003. A statistical model for identifying proteins by tandem mass spectrometry. *Anal. Chem.* **75:** 4646–4658.

Panigrahi, A.K., Gygi, S., Ernst, N.L., Igo Jr., R.P., Palazzo, S.S., Schnaufer, A., Weston, D., Carmean, N., Salavati, R., Aebersold, R., et al. 2001a. Association of two novel proteins, *Tb*MP52 and *Tb*MP48, with the *Trypanosoma brucei* RNA editing complex. *Mol. Cell. Biol.* **21:** 380–389.

Panigrahi, A.K., Schnaufer, A., Carmean, N., Igo Jr., R.P., Gygi, S.P., Ernst, N.L., Palazzo, S.S., Weston, D.S., Aebersold, R., Salavati, R., et al. 2001b. Four related proteins of the *Trypanosoma brucei* RNA editing complex. *Mol. Cell. Biol.* **21:** 6833–6840.

Panigrahi, A.K., Allen, T.E., Haynes, P.A., Gygi, S.P., and Stuart, K. 2003a. Mass spectrometric analysis of the editosome and other multiprotein complexes in *Trypanosoma brucei*. *J. Am. Soc. Mass Spectrom.* **14:** 728–735.

Panigrahi, A.K., Schnaufer, A., Ernst, N.L., Wang, B., Carmean, N., Salavati, R., and Stuart, K. 2003b. Identification of novel components of *Trypanosoma brucei* editosomes. *RNA* **9:** 484–492.

Panigrahi, A.K., Ernst, N.L., Domingo, G.J., Fleck, M., Salavati, R., and Stuart, K.D. 2006. Compositionally and functionally distinct editosomes in *Trypanosoma brucei*. *RNA* **12:** 1038–1049.

Panigrahi, A.K., Zíková, A., Dalley, R.A., Acestor, N., Ogata, Y., Anupama, A., Myler, P.J., and Stuart, K.D. 2008. Mitochondrial complexes in *Trypanosoma brucei:* A novel complex and a unique oxidoreductase complex. *Mol. Cell. Proteom* **7:** 534–545. doi: 10.1074/mcp.M700430-MCP200.

Pelletier, M. and Read, L.K. 2003. RBP16 is a multifunctional gene regulatory protein involved in editing and stabilization of specific mitochondrial mRNAs in *Trypanosoma brucei*. *RNA* **9:** 457–468.

Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29:** e45. doi: 10.1093/nar/29.9.e45.

Sbicego, S., Alfonzo, J.D., Estevez, A.M., Rubio, M.A., Kang, X., Turck, C.W., Peris, M., and Simpson, L. 2003. RBP38, a novel RNA-binding protein from trypanosomatid mitochondria, modulates RNA stability. *Eukaryot. Cell* **2:** 560–568.

Schumacher, M.A., Karamooz, E., Zíková, A., Trantírek, L., and Lukeš, J. 2006. Crystal structures of *Trypanosoma brucei* MRP1/MRP2 guide-RNA-binding complex reveals RNA matchmaking mechanism. *Cell* **126:** 701–711.

Simpson, L., Aphasizhev, R., Gao, G., and Kang, X. 2004. Mitochondrial proteins and complexes in *Leishmania* and *Trypanosoma* involved in U-insertion/deletion RNA editing. *RNA* **10:** 159–170.

Stuart, K. and Panigrahi, A.K. 2002. RNA editing: Complexity and complications. *Mol. Microbiol.* **45:** 591–596.

Stuart, K.D., Schnaufer, A., Ernst, N.L., and Panigrahi, A.K. 2005. Complex management: RNA editing in trypanosomes. *Trends Biochem. Sci.* **30:** 97–105.

Trotter, J.R., Ernst, N.L., Carnes, J., Panicucci, B., and Stuart, K. 2005. A deletion site editing endonuclease in *Trypanosoma brucei*. *Mol. Cell* **20:** 403–412.

Vanhamme, L., Perez-Morga, D., Marchal, C., Speijer, D., Lambert, L., Geuskens, M., Alexandre, S., Ismaïli, N., Göringer, U.H., Benne, R., et al. 1998. *Trypanosoma brucei* TBRGG1, a mitochondrial oligo(U)-binding protein that co-localizes with an in vitro RNA editing activity. *J. Biol. Chem.* **273:** 21825–21833.

Vondrušková, E., van den Burg, J., Zíková, A., Ernst, N.L., Stuart, K., Benne, R., and Lukeš, J. 2005. RNA interference analyses suggest a transcript-specific regulatory role for mitochondrial RNA-binding proteins MRP1 and MRP2 in RNA editing and other RNA processing in *Trypanosoma brucei*. *J. Biol. Chem.* **280:** 2429–2438.

Wang, B.B., Ernst, N.L., Palazzo, S.S., Panigrahi, A.K., Salavati, R., and Stuart, K. 2003. TbMP44 is essential for RNA editing and structural integrity of the editosome in *Trypanosoma brucei*. *Eukaryot. Cell* **2:** 578–587.

Zíková, A., Kopečná, J., Schumacher, M.A., Stuart, K., Trantírek, L., and Lukeš, J. 2008. Structure and function of the native and recombinant mitochondrial MRP1/MRP2 complex from *Trypanosoma brucei*. *Int. J. Parasitol.* doi: 10.1016/j.ijpara.2007.12.009.