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TbMP42, a Protein Component of the RNA Editing Complex in African Trypanosomes, Has Endo-Exoribonuclease Activity

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Summary

RNA editing in trypanosomatids is catalyzed by a high molecular mass RNP complex, which is only partially characterized. TbMP42 is a 42 kDa protein of unknown function that copurifies with the editing complex. The polypeptide is characterized by two Zn fingers and a potential barrel structure/OB-fold at its C terminus. Using recombinant TbMP42, we show that the protein can bind to dsRNA and dsDNA but fails to recognize DNA/RNA hybrids. rTbMP42 degrades ssRNA by a 3′ to 5′ exoribonuclease activity. In addition, rTbMP42 has endoribonuclease activity, which preferentially hydrolyzes non-base-paired uridylate-containing sequences. Gene silencing of *TbMP42* inhibits cell growth and is ultimately lethal to the parasite. Mitochondrial extracts from TbMP42-minus trypanosomes have only residual RNA editing activity and strongly reduced endo-exoribonuclease activity. However, all three activities can be restored by the addition of rTbMP42. Together, the data suggest that TbMP42 contributes both endo- and exoribonuclease activity to the editing reaction cycle.

Introduction

The RNA editing reaction of mitochondrial mRNAs in kinetoplastid protozoa is characterized by an enzymatic reaction cycle that inserts and deletes uridylate nucleotides into otherwise incomplete primary transcripts. The process is catalyzed by a high molecular mass ribonucleoprotein complex, which is composed of preedited mRNAs, guide (g) RNAs, and an uncertain number of proteins (Madison-Antenucci et al., 2002; Worthey et al., 2003; Simpson et al., 2004). Depending on the enrichment protocol, active RNA editing complexes contain as little as 7 (Rusché et al., 1997), 13 (Aphasizhev et al., 2003a), or up to 20 polypeptides (Panigrahi et al., 2001).

Although not all contributing enzyme activities of a full reaction cycle are currently known, it is generally accepted that the initiation step of the process involves the formation of an antiparallel RNA/RNA duplex structure between the preedited mRNA and a cognate gRNA molecule. It is assumed that the base pairing interaction is catalyzed by the RNA annealing factors gBP21 and gBP27, which have been identified in *Trypanosoma brucei*, *Leishmania tarentolae*, and *Crithidia fasciculata* (Müller et al., 2001; Blom et al., 2001; Aphasizhev et al., 2003b). The gRNA/pre-mRNA duplex positions an editing site 5′ of the helical element, thereby defining the endoribonucleolytic cleavage site of the preedited mRNA. An endoribonucleolytic enzyme activity has been identified in editing-active mitochondrial fractions (Adler and Hajduk, 1997; Piller et al., 1997; Salavati et al., 2002); however, no candidate protein has yet been characterized. During deletion-type RNA editing, uridylate residues are exonucleolytically removed from the 3′ end of the 5′ mRNA cleavage product and released as UMP. This requires a U-specific 3′ to 5′ exoribonuclease (exoUase). As for the endoribonuclease, mitochondrial extracts contain exoUase activity (Aphasizhev and Simpson, 2001, Igo et al., 2002), but no candidate protein has been identified to date. Insertion-type editing requires the addition of U nucleotides to the 3′ end of the 5′ mRNA cleavage product. This reaction step is catalyzed by a 3′ terminal uridylyl transferase (TUTase). The enzyme has recently been cloned from both *Leishmania* and trypanosomes and was characterized as a member of the DNA polymerase β superfamily of nucleotidyltransferases (Aphasizhev et al., 2003c; Ernst et al., 2003).

An editing reaction cycle is completed by the ligation of the processed 5′ fragment to the 3′ fragment of the pre-mRNA. Two editing-specific RNA ligases (REL1, REL2) have been identified and were biochemically and genetically characterized (McManus et al., 2001; Schnaufer et al., 2001; Huang et al., 2001).

Aside from these core activities, evidence exists that several auxiliary factors add to the reaction cycle. Among these factors are proteins which interact and stabilize preedited mRNA, such as REAP1 (Madison-Antenucci and Hajduk, 2001), polypeptides which can bind to the 3′ oligo(U) extensions of gRNAs (TbRGG1) (Vanhamme et al., 1998), or proteins such as mHel61p, a complex-associated putative RNA helicase (Missel et al., 1997; Stuart et al., 2002), which may catalyze the unwinding of fully base-paired gRNAs from edited mRNAs.

Potential candidates for the yet unidentified catalytic components of the editing machinery are proteins that copurify with the complex (Stuart et al., 2002; Worthey et al., 2003; Simpson et al., 2004). They include polypeptides that have been shown to contain Zn finger motifs, suggesting direct contact points to nucleic acid ligand molecules (Panigrahi et al., 2001, Huang et al., 2002; Lu et al., 2003). One such protein is TbMP42. The mitochondrial polypeptide has a molecular mass of 42 kDa and was first identified in African trypanosomes (Panigrahi et al., 2001). It shares sequence homology to three other *T. brucei* Zn finger proteins (TbMP81, TbMP63, and TbMP18), although the four polypeptides

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show no sequence homology to other polypeptides. A TbMP42-specific monoclonal antibody was shown to immunoprecipitate deletion and insertion RNA editing activity (Panigrahi et al., 2001), supporting the evidence that the polypeptide is associated with the editing complex.

Here we demonstrate that recombinant TbMP42 binds to dsRNA and dsDNA and has both endoribonuclease and 3′ to 5′ exoribonuclease activity. The endoribonuclease activity acts preferentially on looped-out uridylate residues, and the exoribonuclease activity terminates on RNA duplex structures. Further, we show that gene silencing of *TbMP42* is lethal for the parasite and that TbMP42-minus cells have only residual RNA editing activity. However, the editing deficiency can be rescued by the addition of exogenous rTbMP42, which provides evidence for an involvement of the protein during the editing reaction cycle.

Results

Recombinant TbMP42 Binds ds Nucleic Acids

We cloned *TbMP42* by rapid amplification of cDNA ends (RACE). Using the cDNA sequence information, we amplified the genomic copy of *TbMP42* and sequenced the resulting open reading frame (ORF). The ORF is 1179 bp in length and codes for a 393 amino acid polypeptide. A Southern blot analysis revealed that *TbMP42* is encoded by a single-copy gene, and by RT-PCR we determined that the transcript is expressed at equal levels in both major life cycle stages of the parasite (data not shown).

In order to characterize the biochemical properties of TbMP42, we constructed a plasmid-encoded $(his)_6$-tagged version of *TbMP42*. The plasmid was transformed into *E. coli* M15[pREP4] bacteria, and upon induction with isopropylthiogalactoside (IPTG), high amounts of recombinant (r) TbMP42 were expressed (Figure 1A). Since the induction of rTbMP42 caused the formation of inclusion bodies within the bacterial cells, we used inclusion body preparations for the purification of the polypeptide. All purification steps were performed at denaturing conditions (8 M urea) and started with a Ni-chelate affinity chromatography step utilizing the $(his)_6$-tag of the recombinant protein. rTbMP42-containing fractions were further purified by anion exchange chromatography, in some cases followed by isoelectric focusing or dye binding chromatography. The resulting urea-containing protein preparations were dialyzed and characterized in SDS-containing polyacrylamide gels (Figure 1A). Recombinant $(his)_6$-tagged TbMP42 migrated as a homogenous protein population with the expected electrophoretic mobility of a 43 kDa polypeptide. The presence of folded protein domains was monitored by circular dichroism (CD) measurements (Figure 1B), and the spectra indicated that the protein preparations were essentially unstructured. However, the addition of Zn^{2+} cations induced a folding reaction resulting in protein preparations with 50%–60% α-helical content and 10%–20% β sheet structure (Figure 1B). The critical Zn^{2+} concentration for refolding was determined as $\geq$0.1 mM.

The ability of refolded rTbMP42 to interact with nucleic acid ligands was measured in real-time resonant mirror experiments. rTbMP42 was covalently coupled to an amino silane biochip surface and incubated with different nucleic acid ligands. Figure 1C shows a representative set of binding curves for a 15 bp double-stranded (ds) RNA ligand at different concentrations.

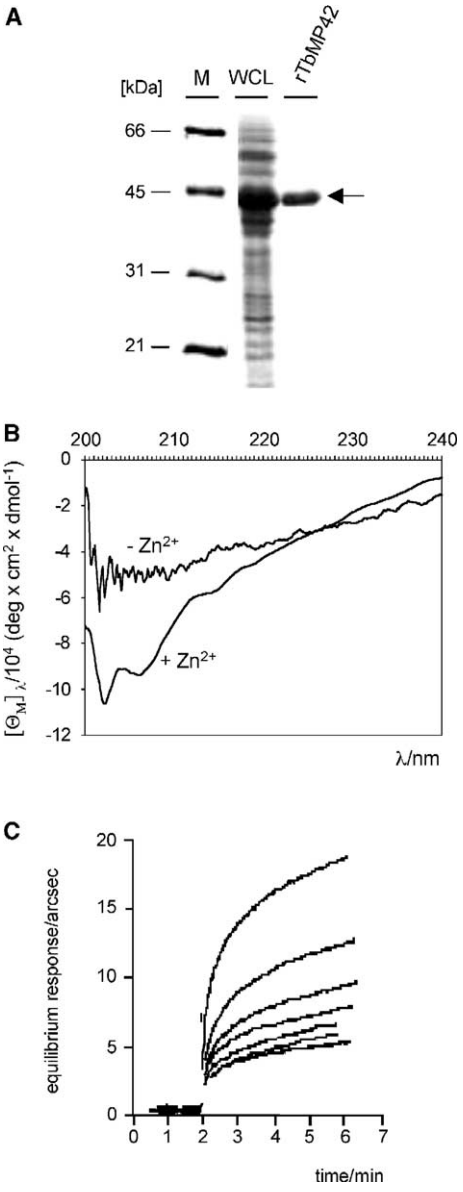

Figure 1. Structural Characterization and RNA Binding Analysis of rTbMP42

(A) SDS-PAGE of a whole-cell protein lysate (WCL) from rTbMP42-expressing *E. coli* in comparison to purified rTbMP42. M, marker proteins.

(B) CD spectra of renatured rTbMP42 (2.6 μM) in the presence and absence of Zn^{2+} cations (0.1 mM). The spectrum of the folded protein corresponds to 50%–60% α-helical content and 10%–20% β sheet structures.

(C) Real-time monitoring of the concentration-dependent binding of a 15 bp dsRNA ligand (bottom to top: 2, 5, 10, 16, 32, 50, and 100 nM) to rTbMP42.

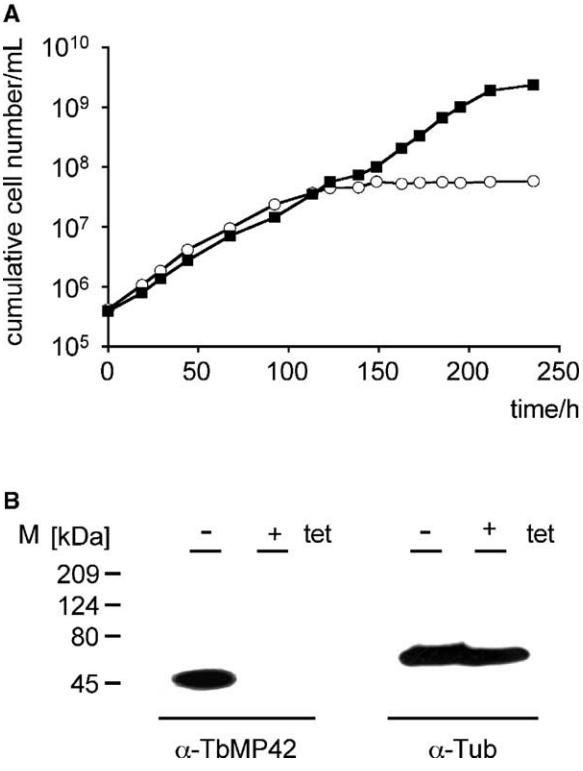

Figure 2. Phenotypic and Molecular Analysis of TbMP42-Minus Trypanosomes

(A) Growth behavior of a clonal *TbMP42*-RNAi trypanosome cell line in the presence (open circles) and absence (filled squares) of tetracycline (tet).

(B) Western blot analysis for TbMP42 and α-tubulin in tet-induced (+tet) and noninduced (–tet) trypanosomes.

The K_d for the dsRNA/rTbMP42 interaction was calculated as 10 nM, and binding equilibrium was reached within 2–4 min. rTbMP42 was also capable of binding to a 18 bp dsDNA ligand to ssDNA (15–18 nt) but failed to recognize a 18 bp DNA/RNA hybrid (data not shown).

TbMP42-Minus Cells Are Not Viable

To identify the mitochondrial function of TbMP42 we performed a gene knockdown experiment by RNA interference (RNAi). A 701 bp fragment of the coding region of *TbMP42* was cloned into the RNAi vector pZJM (Wang et al., 2000). The resulting plasmid was linearized and used to transfect insect stage *T. brucei* 29-13 parasites (Wirtz et al., 1999). Ble-resistant transfectants were cloned, and the synthesis of *TbMP42*-specific dsRNA was induced by the addition of tetracycline (tet) to the culture medium. Figure 2A shows a representative growth curve of a clonal *TbMP42* RNAi cell line in the absence and presence of tet. While noninduced parasites grew with a normal doubling time, tet-induced cells showed a severe growth rate phenotype. The parasites stopped multiplying around 120 hr after the addition of tet and eventually died. A molecular analysis of the phenotype revealed that in as little as 48 hr after the induction with tet, both *TbMP42*-specific mRNA (data not shown) and TbMP42 protein (Figure 2B) were below the level of detection.

TbMP42-Minus Cells Show Reduced RNA Editing Activity

In order to test whether the described gene knockdown phenotype of TbMP42-minus *T. brucei* was correlated with a deficiency of the parasites to perform RNA editing, we analyzed the processing reaction directly. For that we used mitochondrial detergent extracts from both *T. brucei* cells that express TbMP42 and parasites that were treated with tet for 72 hr and therefore lack the protein. The extracts were separated by centrifugation in glycerol density gradients and fractionated. All fractions were assayed for their in vitro RNA editing activity using a precleaved U-insertion assay (Igo et al., 2000). The activity sedimented in both samples as a broad peak of approximately 20S–35S (Figures 3A and 3B). However, the peak fraction derived from the TbMP42-minus cells showed a strongly reduced RNA editing activity of only 10%. This indicated that the absence of TbMP42 severely impacts the editing reaction, though does not totally abolish it. The data further suggest that the absence of the protein does not result in a significant structural rearrangement or even disassembly of the editing machinery. This was further confirmed by analyzing the gradient distribution of the two editing-specific RNA ligases REL1 and REL2 (McManus et al., 2001; Schnaufer et al., 2001; Huang et al., 2001), which was identical in TbMP42-plus and TbMP42-minus cells (data not shown).

Last, we analyzed the capacity of TbMP42-minus cells to perform the editing reaction in vivo. This was done by poisoned primer extension experiments testing the abundance of edited apocytochrome b (Cyb) and NADH dehydrogenase subunit 7 (ND7) transcripts in steady state RNA-preparations from TbMP42-minus and TbMP42-plus cells (Figure 3C). In agreement with the above described in vitro data, the downregulation of *TbMP42* (for 72 hr) had a negative effect on the abundance of the two edited mRNAs, although the cells were still capable of performing the processing reaction to some degree (5%–15%).

Exogenous TbMP42 Rescues the Editing Deficiency of TbMP42-Minus Cells

Based on the described result, we asked whether the addition of rTbMP42 might be able to rescue the reduced editing activity of TbMP42-minus cells. This was experimentally addressed by performing in vitro U-insertion editing reactions with a mitochondrial fraction from TbMP42-minus cells. As shown above, the fraction had a reduced editing activity of only 10% (Figure 4A). Individual samples were supplemented with increasing concentrations of rTbMP42 (0.05–6 ng/μl), and as shown in Figure 4A, rTbMP42 was capable of rescuing the editing deficiency in a concentration-dependent fashion. At a concentration of 6 ng/μl, the reaction reached its maximal level, which was in the range of 90% of the value of a fraction that contains endogenous TbMP42 (Figure 4B). The amount of editing complex-associated rTbMP42 was determined by reisolating rTbMP42-supplemented complexes by density centrifugation fol-

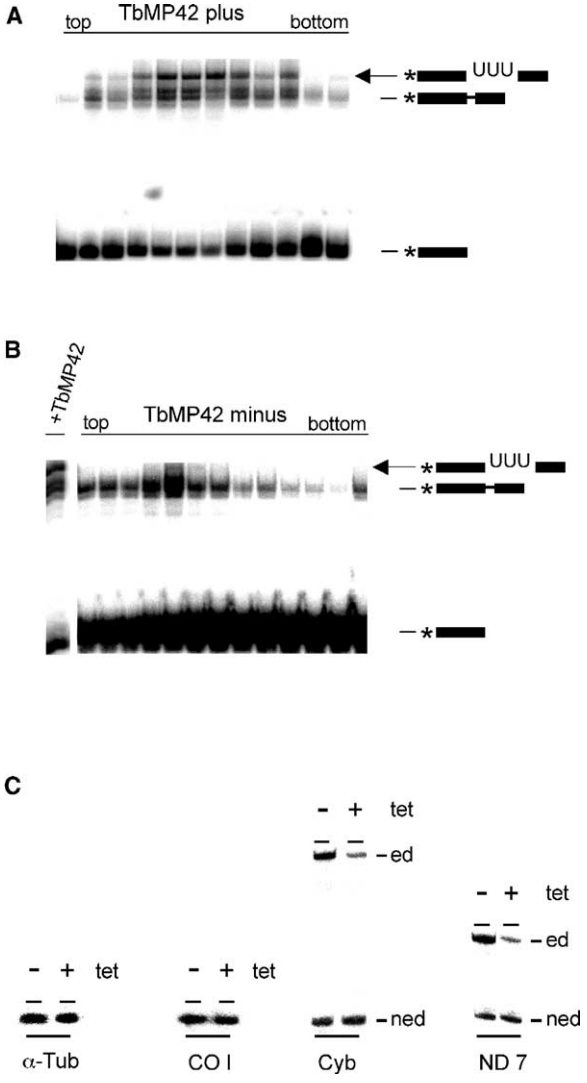

Figure 3. In Vitro RNA Editing Analysis of TbMP42-Minus Trypanosomes

Mitochondrial detergent extracts were separated in isokinetic glycerol gradients and fractionated. Each fraction was tested for its RNA editing activity. The electrophoretic mobilities of the editing product, the ligation product and the pre-mRNA 5′ fragment are given on the right (top to bottom). An asterisk represents the position of the radioactive label. (A) In vitro RNA editing activity of mitochondrial extracts from TbMP42-plus trypanosomes and (B) from TbMP42-minus parasites. Top and bottom of the gradients are marked accordingly. (C) Poisoned primer extension analysis of four mRNAs from the TbMP42-RNAi cell line 72 hr after tet induction: α-Tub, α-tubulin; COI, cytochrome oxidase I; Cyb, apocytochrome b; ND7, NADH dehydrogenase subunit 7. Extension products representing the edited (ed) and nonedited (ned) versions of the CYb and ND7 mRNAs are indicated. α-Tub is a nuclear transcript and COI is a never edited mitochondrial transcript.

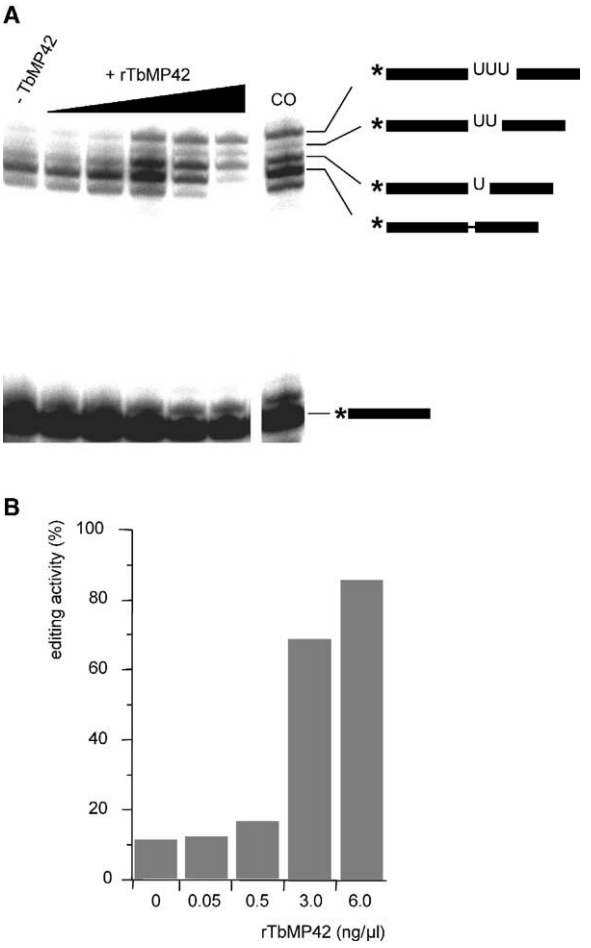

Figure 4. rTbMP42 Is Capable of Rescuing the Editing Deficiency of Mitochondrial Extracts from TbMP42-Minus Trypanosomes

(A) Autoradiogram of a precleaved RNA editing in vitro assay. –TbMP42 represents a sample from TbMP42-minus cells, and CO represents a control reaction using a mitochondrial extract from TbMP42-plus cells. The electrophoretic mobilities of the editing product, two editing intermediates, the nonproductive ligation product, and the pre-mRNA 5′ fragment are given on the right (top to bottom). An asterisk represents the position of the radioactive label.

(B) Quantitative analysis of the signal of the editing product shown in (A).

lowed by Western blotting. The data showed that 80% of rTbMP42 was complex associated (data not shown).

Recombinant TbMP42 has 3′ to 5′ Exoribonuclease and Endoribonuclease Activity

Based on the above-described finding that rTbMP42 was able to interact with ds nucleic acids, we investigated the fate of a synthetic RNA editing substrate upon incubation with rTbMP42. The molecule was termed U5-hybrid RNA (Figure 5A) and represents a gRNA/preedited mRNA hybrid molecule specific for a deletion-type RNA editing reaction. The editing domain is defined by five looped-out uridylate (U) residues, which are flanked by two 13 bp helices formed between the gRNA (bottom strand) and the preedited mRNA (top strand). Figure 5B shows a representative result using a U5-hybrid RNA preparation in which the pre-mRNA was radioactively labeled at its 5′ end. While the mock-treated sample was stable over the entire incubation period (120 min), the addition of 7.5 ng/μl rTbMP42 resulted in the appearance of four pre-mRNA cleavage products varying in length from 16 to 13 nt (Figure 5B). Thus, rTbMP42 induced a partial ribonucleolytic degra-

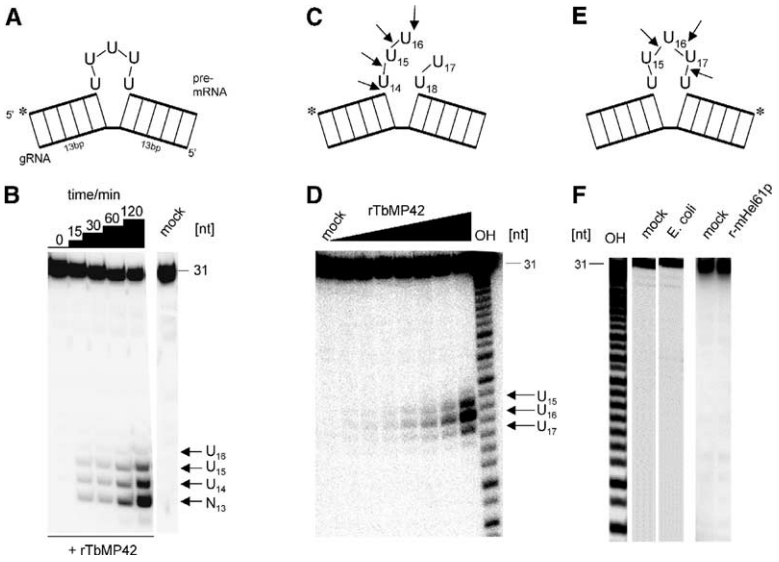

Figure 5. Exo- and Endoribonucleolytic Hydrolysis of an RNA Editing Model Substrate by rTbMP42

(A) Graphical representation of a pre-mRNA/gRNA hybrid molecule with five single-stranded uridylate residues flanked by two 13 bp stem structures (U5 hybrid RNA). An asterisk represents the position of the radioactive label. (B) Time-dependent hydrolysis of U5-hybrid RNA by rTbMP42. A representative autoradiograph of a separation of the exoribonucleolytic hydrolysis products in a denaturing polyacrylamide gel is shown. Hydrolysis positions (U16–N13) are marked by arrows and are graphically represented in (C). (D) Endoribonucleolytic hydrolysis of U5-hybrid RNA (the radioactive label [*] is located at the 3′ end of the pre-mRNA). Hydrolysis products are separated by denaturing PAGE and are marked by arrows (U17–U15). A graphical representation is shown in (E). OH represents an alkaline hydrolysis ladder of 5′ radioactively labeled input RNA. (F) Ribonucleolytic hydrolysis of U5-hybrid RNA with unrelated proteins or protein extracts. *E. coli*, incubation of U5-hybrid RNA with a protein extract from *E. coli* cells; r-mHel61, incubation of U5-hybrid RNA with his-tagged *T. brucei* mHel61. Both protein samples were treated identically to the purification protocol for rTbMP42.

dation of the pre-mRNA of U5-hybrid RNA which was suggestive of a two-step scenario: First, an endoribonucleolytic cleavage at position U16 of the pre-mRNA and, second, a 3′ to 5′ exoribonucleolytic trimming reaction of the 5′ cleavage product (Figure 5C). A comparison of the signal intensities of the different hydrolysis fragments at early time points versus late time points suggested a distributive reaction type which is terminated at position 13, the next base-paired nucleotide within the pre-mRNA sequence (Figure 5C). Although we identified in all of our experiments some minor degradation (<1%) into the second helix of U5-hybrid RNA (position 12 in Figure 5B), this can be attributed to a breathing reaction and/or alternative secondary structure at the helical end.

In order to experimentally confirm the initial endoribonucleolytic reaction step, we used a U5-hybrid RNA preparation that contained a radioactively 3′ end labeled pre-mRNA molecule. Upon incubation with rTbMP42, the RNA was predominantly hydrolyzed at the anticipated position (U16, >90%), in addition to some minor cleavage at the two surrounding nucleotides U15 and U17 (Figures 5D and 5E). Thus, rTbMP42 shows characteristics of a structure-specific endonuclease, which specifically recognizes looped-out nucleotides.

To exclude the possibility that the two identified ribonucleolytic activities stem from copurifying *E. coli* ribonucleases, we tested an identical column fraction derived from the parental *E. coli* M15[pREP4] strain that did not contain the *rTbMP42* expression plasmid. This fraction was free of any nucleolytic activity (Figure 5F). Furthermore, we analyzed another recombinant $(his)_6$-tagged protein preparation to eliminate the possibility of copurifying *E. coli* ribonucleases through protein/protein interaction (despite the presence of 8 M urea throughout the purification). For this we chose mHel61p, which represents another editing complex-associated protein (Missel et al., 1997, Stuart et al., 2002). As above, his-tagged recombinant mHel61p preparations showed no ribonucleolytic cleavage activity (Figure 5F). Lastly, we tested whether the gRNA molecule in U5-hybrid RNA was hydrolyzed by rTbMP42. However, even a 3 hr incubation with 7.5 ng/μl rTbMP42 did not result in any detectable nucleolytic degradation (data not shown). Thus, the rTbMP42-mediated cleavage of U5-hybrid RNA is specific for the pre-mRNA of the gRNA/pre-mRNA hybrid and starts with an endoribonuclease reaction step followed by a 3′ to 5′ exoribonucleolytic degradation of the 5′ cleavage product.

TbMP42-Minus Cells Show Reduced Endo-Exoribonuclease Activity

In order to test whether the two identified nucleolytic activities of rTbMP42 can be correlated to activities of the RNA editing reaction cycle, we analyzed the cleavage pattern of U5-hybrid RNA by an editing-active mitochondrial fraction (EAF) from wild-type trypanosomes. As before, by using complementary radioactive labeling strategies for U5-hybrid RNA, we were able to analyze both ribonucleolytic activities. Figure 6A shows that wild-type EAF contained endoribonuclease activity that cleaved U5-hybrid RNA at the same position as rTbMP42 (U16>U15>U17). Importantly, a mitochondrial extract from the *TbMP42*-RNAi knockdown strain showed only residual endoribonuclease activity (<5%), suggesting that the majority of the wild-type activity is due to the presence of TbMP42 (Figure 6A). Adding back rTbMP42 to the RNAi extract fully restored the activity (Figure 6A).

Identical experiments were performed to analyze the exoribonucleolytic activity with essentially the same result (Figure 6B). Editing-active fractions from wild-type trypanosomes showed 3′ to 5′ exoribonucleolytic activity and cleaved U5-hybrid RNA at the same nucleotides as rTbMP42 (U16, U15, U14, and N13). The exoribonucleolytic activity was strongly reduced (to ~5%) in the TbMP42-minus RNAi strain but was completely rescued by the addition of rTbMP42 (Figure 6B).

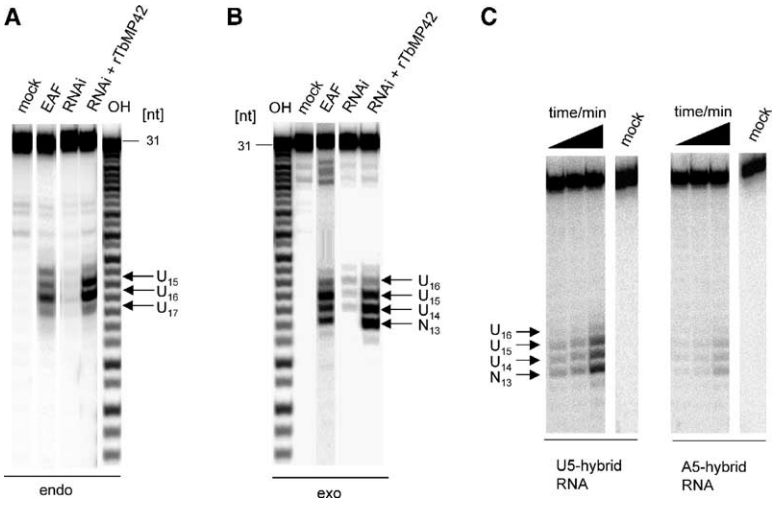

Figure 6. Endo- and Exoribonucleolytic Activity of Mitochondrial Extracts from TbMP42-Plus and TbMP42-Minus Parasites

To assay for the two ribonucleolytic activities, the pre-mRNA of U5-hybrid RNA was radioactively labeled either at its 3′ end (A) or the 5′ end (B). The RNA preparations were incubated with glycerol gradient fractions from TbMP42-plus cells (EAF) or from a *TbMP42* knocked down RNAi cell line (RNAi). RNAi + rTbMP42 represents a sample where rTbMP42 was added back to the RNAi fraction. Hydrolysis products were separated in denaturing polyacrylamide gels and are marked by arrows. OH represents an alkaline hydrolysis ladder of 5′ radioactively labeled input RNA. (C) Uridylate preference of the endo/exoribonucleolytic activity of rTbMP42. Kinetic (10, 15, 30 min) of the rTbMP42-mediated hydrolysis of U5-hybrid RNA in comparison to A5-hybrid RNA. Hydrolysis positions are marked by arrows (U16–N13).

The Ribonucleolytic Activity of rTbMP42 Has a Preference for U Nucleotides

Experiments with partially purified mitochondrial extracts from *T. brucei* have shown that the exoribonucleolytic reaction step of the editing process is a U nucleotide-specific process (Cruz-Reyes and Sollner-Webb, 1996; Lawson et al., 2001; Aphasizhev and Simpson, 2001; Igo et al., 2002). Therefore, we analyzed whether the ribonucleolytic cleavage reactions of rTbMP42 have U-specific characteristics. This was tested by comparing the rTbMP42-induced cleavage of U5-hybrid RNA to another synthetic RNA that contained five looped out A nucleotides instead of the five Us (Figure 6C). The appearance of the characteristic hydrolysis products indicated that both pre-mRNAs were endoribonucleolytically cleaved and that the resulting 5′ fragment was subsequently trimmed by the 3′–5′ exoribonuclease activity. However, a quantitative comparison demonstrated that the A substrate was hydrolyzed to a significantly lesser degree, in the range of only 5%–10% of the U RNA. Thus, while rTbMP42 can act on U and A nucleotides, the U nucleotide-containing pre-mRNA is the preferred substrate.

The Ribonucleolytic Activities of rTbMP42 Reside within Its C-Terminal Half

TbMP42 contains, with its two Zn fingers and a potential C-terminal barrel structure/OB-fold, three protein domains known to interact with nucleic acid ligands (Lu et al., 2003, Theobald et al., 2003). This led us to test whether all three motifs are required for the ribonucleolytic activities of the protein. We constructed two truncated rTbMP42 mutants: first, an N-terminal (NT) variant (amino acids [aa] 1–250) that contains both zinc finger motifs but lacks the potential barrel/OB-fold; second, a C-terminal (CT) protein variant (aa 251–393) that lacks the two zinc fingers but contains the barrel/OB-fold (Figure 7A). Both mutant polypeptides were expressed as $(his)_6$-tagged proteins and were purified following the same procedure as outlined for full-length rTbMP42 (Figure 7B). The two polypeptides were tested for their endo/exoribonuclease activity using U5-hybrid RNA as a substrate, and the results are shown in Figure 7C. While the Zn finger-containing N-terminal polypeptide showed no ribonucleolytic activity, the C-terminal fragment still contained both activities. This suggests that the two Zn fingers do not contribute to RNA substrate binding and indicates a role for the C-terminal barrel/OB-fold in both RNA recognition and hydrolysis.

Discussion

In this study we describe experiments aimed at identifying a molecular function for TbMP42, a protein component of the RNA editing complex in African trypanosomes and *Leishmania tarentolae* (Panigrahi et al., 2001; Aphasizhev et al., 2003a). We characterized the protein as an endo-exoribonuclease, which degrades ssRNA with a 3′ to 5′ directionality and cleaves RNA molecules endonucleolytically at looped-out nucleotides. The exoribonuclease activity is distributive, it stops at RNA duplex structures, and it has a preference for U nucleotide-containing RNAs over A nucleotide-containing RNAs. Together, these data suggest that TbMP42 contributes to endo- and exoribonucleolytic reaction steps of the RNA editing cycle.

In support of this hypothesis we were able to show that a recombinant, $(his)_6$-tagged preparation of TbMP42 can rescue the reduced editing efficiency of mitochondrial fractions from an epigenetic *TbMP42* knockdown *T. brucei* strain. The restoration was concentration dependent and reached a maximal level of 90% of the in vitro editing activity of a wild-type mitochondrial extract. Thus, rTbMP42 is necessary and sufficient for the restitution of the editing activity in TbMP42-minus mitochondrial extracts. However, it should be noted that due to the precleaved nature of the pre-mRNA substrate, the in vitro assay only monitors the exoribonucleolytic activity of TbMP42 (Igo et al., 2000). Therefore, full-round in vitro editing assays have to be performed to verify the result for both activities at the same time.

Further support for the ribonucleolytic activities of TbMP42 comes from the finding that editosome-con-

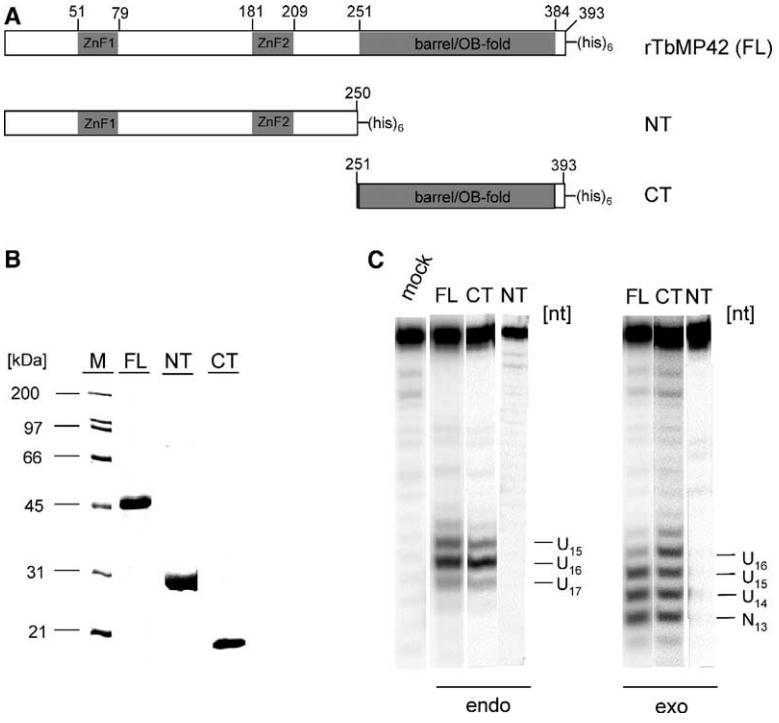

Figure 7. The Ribonucleolytic Activities Are Located within the C-Terminal Fragment of rTbMP42

(A) Schematic representations of the three recombinant proteins: FL, full-length rTbMP42; NT, N-terminal fragment (27 kDa); CT, C-terminal fragment (16 kDa). Each polypeptide carries a C-terminal extension of six histidines $(his)_6$ for affinity purification. The positions of the two zinc finger domains are marked as ZnF1 and ZnF2.

(B) SDS-PAGE of the recombinant proteins after affinity purification. M, marker proteins.

(C) Incubation of U5-hybrid RNA with FL, CT, and NT. Hydrolysis products were separated in denaturing polyacrylamide gels and are marked by arrows. endo, endoribonuclease assay; exo, exoribonuclease assay.

taining mitochondrial fractions from TbMP42-minus trypanosomes have only residual endo-exoribonuclease activity. As before, the addition of rTbMP42 was able to complement this deficiency, which verified that the two ribonucleolytic activities of editosome-containing protein fractions are by and large due to TbMP42. Unfortunately, since the concentration of RNA editing complexes within these fractions is unknown, one cannot deduce any stoichiometric values. Whether one or more rTbMP42 molecules bind to the editing complex remains unclear. However, since editing complexes from TbMP42-minus trypanosomes apparently do not disassemble and are characterized by an apparent S value similar to complexes enriched from TbMP42-plus cells, it is unlikely that many TbMP42 molecules are part of an active RNA editing complex. Rather, the data are suggestive of a structural situation where only one or a few TbMP42 molecules are localized close to the surface of the editing complex. This is supported by the observation that editing complex-associated TbMP42 is accessible for TbMP42-specific antibodies which have been used to immunoprecipitate the entire complex (Panigrahi et al., 2001). It is further supported by the fact that recombinant TbMP42 assembles into TbMP42-minus editing complexes without any activation or preassembly step.

Whether the protein binds to the editing machinery by protein/protein interactions, by RNA/protein interactions, or a combination of both cannot be deduced from the presented data. However, since TbMP42 contains two zinc finger domains, which are not required for the ribonucleolytic activities of the protein, it is tempting to speculate that the Zn fingers function as protein/protein interaction sites. This has been shown for Zn finger proteins in other systems (Rodgers et al., 1996; Kuroda et al., 1996) and has recently been experimentally demonstrated for TbMP63, another Zn finger protein of the RNA editing complex (Kang et al., 2004).

Based on the position of the identified endonucleolytic cleavage sites of the tested RNA editing model substrate and the fact that rTbMP42 is capable of binding to dsRNA, the most plausible RNA binding motif for TbMP42 seems to be the anchor helix of the pre-mRNA/gRNA hybrid. On the protein level the RNA interaction domain lies within the C-terminal half of rTbMP42. Different structure prediction algorithms calculate a barrel structure for this part of the protein, possibly an oligonucleotide/oligosaccharide binding (OB)-fold (Murzin, 1993; Theobald et al., 2003). OB-folds are characterized by a five-stranded β sheet coiled to form a closed β barrel that is capped by an α helix. The motif has been shown to provide a non-sequence-specific binding platform for single-stranded and double-stranded nucleic acids through stacking interactions between aromatic amino acid side chains and heterocyclic bases of the bound ligand. This is consistent with our experimental data, which identified binding to different nucleic acid ligands.

Binding of rTbMP42 to dsRNA and dsDNA was dependent on the presence of Zn^{2+} cations. This was, at least in part, due to a refolding reaction of the recombinant protein which was visualized in real-time resonant mirror experiments and further experimentally confirmed by CD measurements. Since the two Zn fingers are dispensable for the ribonucleolytic activities of rTbMP42, this suggests that defined Zn^{2+} binding sites outside of the two Zn fingers likely act as folding nuclei for the proper folding of the entire protein. Within this context it is important to note that a search for known endo- and exoribonucleolytic protein motifs within

TbMP42 was unsuccessful. Thus, the polypeptide might rely on so-far uncharacterized protein domains for its ribonucleolytic activities. On the other hand, there is very limited sequence homology among the exoribonuclease superfamilies (Zuo and Deutscher, 2001), and some exoribonucleases as well as DNA endo-exonucleases have been shown to contain multiple invariant acidic residues, which are involved in metal ion binding (Sayers and Artymiuk, 1998; Zuo and Deutscher, 2001; Feng et al., 2004). This is consistent with the fact that all known exoribonucleases require divalent cations for their activity, and two metal ion catalysis is probably a common feature of exonucleases (Steitz and Steitz, 1993; Zuo and Deutscher, 2001). As a consequence, it seems feasible that the rTbMP42-bound Zn^{2+} cations are not only required for the proper folding of the protein but also for the nucleolytic hydrolysis reactions, especially in the context that DNA-specific endo-exonucleases have been found to be Zn-dependent enzymes (Fraser, 1994). TbMP42 contains 34 acidic amino acids, and we determined a critical Zn^{2+} concentration of 0.1 mM in order to convert unfolded rTbMP42 into active protein. Lastly, we cannot exclude that TbMP42, within the context of the assembled editing complex, has a preference for only one of its ribonucleolytic activities. A similar scenario has been described for mutants of bovine pancreatic ribonuclease A (Cuchillo et al., 2002).

Gene silencing of *TbMP42* stops the parasites from multiplying and leads to cell death after a few days. Therefore, TbMP42 must be considered a required component for cell survival. However, mitochondrial extract in which both TbMP42 protein and the transcript for the polypeptide are below the level of detection still show about 10% in vitro editing activity and also show edited mRNAs (although at a significantly reduced level). This indicates that TbMP42 is an important component of the editing reaction cycle, but it is not essential. One can speculate that in the absence of TbMP42 other ribonucleases of the editing complex can substitute for the lack of the protein, especially since molecular redundancy has been shown for several other editing components (for an overview, see Simpson et al., 2004). Candidate proteins might be the above-described TbMP42-related Zn finger proteins TbMP81 and TbMP63 (Panigrahi et al., 2001) or TbMP99 and TbMP100, which have been shown to contain endo-exonuclease phosphatase motifs (Simpson et al., 2004). In addition, experimental evidence exists that suggests that the molecular architecture of the editing complex involves two different subcomplexes, which physically separate the U-deletion and the U-insertion reaction. This might provide a rational why more than one nuclease activity is required (Schnaufer et al., 2003).

In summary, our analysis identified rTbMP42 as a 3′ to 5′ exoribonuclease as well as an endoribonucleolytic enzyme. The protein functions as part of the RNA editing complex, which suggests that the endonuclease activity contributes to the nucleolytic cleavage of the preedited mRNA around an editing site and that the exoribonucleolytic activity is used for the trimming reaction of the U extensions of the 5′ cleavage product. Thus, TbMP42 represents a candidate polypeptide for two important activities of the editing reaction cycle.

Experimental Procedures

Trypanosome Cell Growth

The insect life cycle stage of *Trypanosoma brucei* 427 (Cross, 1975) and strain 29-13 (Wirtz et al., 1999) was grown at 27°C in SDM-79 medium supplemented with 10% (v/v) heat-inactivated bovine fetal calf serum (FCS) (Brun and Schönenberger, 1979). Parasite cell densities were determined by automated cell counting.

Cloning of *TbMP42*

TbMP42 was cloned by rapid amplification of cDNA ends (RACE). Total RNA was isolated from 3×10^9 *T. brucei* cells and used for the isolation of poly(A)$^+$ RNA using oligo $(dT)_{25}$ latex beads. A hundred nanograms of poly(A)$^+$ RNA was incubated with 50 μM of an oligo $(dT)_{27}$ primer for 45 min at 42°C in the presence of 10 U M-MuLV reverse transcriptase (RT) for the synthesis of cDNA. Samples were RNase H digested, phenol/chloroform extracted, and after ethanol precipitation dissolved in 20 μl TE (pH 8.0). The cDNA preparation was used as template for the RACE amplification using the following primers: 3′-RACE primers: GAYGGIGARTGRTTYYTIGTIACIGG and TNGARGARG-TIAAYCCIGARGARATIAA; 5′-RACE primers: GAA CAGTTTCTGTACTATATTG, AGAGGGTCCCTCGAAGTCTGTG, and GTGCTTCGCCTGGT-AATGGTGTTG. The 5′ RACE and 3′ RACE products were sequenced, and the sequence information used to PCR amplify the full-length *TbMP42* gene from *T. brucei* genomic DNA using primers TbMP42-5′, CGCACCTGAGGAGGGTGAAG TGG, and TbMP42-3′, AGAGGGTCCCTCGAAGTCTGTG. The PCR product was cloned into pBS SK$^-$ (Stratagene), and the nucleotide sequence of both strands of the insert was determined by automated sequencing.

Purification of rTbMP42

Full length *TbMP42* was amplified from genomic DNA using primers CGTCATGAAGCGTGTTACTTCACATATTTC and GAAGATCTC ACCCTCAACACTGACCCACAG. The PCR product was cloned into plasmid pQE60 (Qiagen). Two truncated versions of *TbMP42* were generated by restriction endonuclease hydrolysis of the PCR product. The DNA sequences translate into a N-terminal (aa 1–250) and a C-terminal variant (aa 251–393) of TbMP42. All three constructs were transformed into *E. coli* M15pREP4 (Qiagen). Positive clones were verified by DNA sequencing. Protein expression was performed in 1 liter bacterial cultures, induced by the addition of 1 mM isopropylthiogalactoside (IPTG) at an OD_{600} of 0.5–0.6. Cells were grown for an additional 3 hr and harvested. The proteins were isolated from inclusion bodies in lysis buffer (10 mM Tris/HCl [pH 8.0], 0.1 M NaH_2PO_4, 8 M urea). Lysates were loaded onto a Ni^{2+} chelating column, and bound proteins were eluted using a pH step gradient (pH 6.3; pH 5.9; pH 3.0). rTbMP42-containing fractions were further purified by anion exchange chromatography at denaturing conditions (8 M urea), in some cases followed by isoelectric focusing and dye binding chromatography. Purified rTbMP42 preparations were proteolytically cleaved, and the resulting peptide sequences were analyzed by mass spectroscopy (MALDI-TOF). No contaminating peptides from *E. coli* ribonucleases were identified. Denatured rTbMP42 was refolded by dialysis at 4°C against 20 mM HEPES (pH 7.5), 30 mM KCl, 10 mM $Mg(OAc)_2$, 5 mM $CaCl_2$, 1 mM $ZnSO_4$. The percentage of active rTbMP42 was determined in ligand binding experiments using a resonant mirror system (see below). Typically about 5% of the recombinant protein preparations were capable of binding to ds nucleic acids. CD spectra were recorded at a protein concentration of 0.12 mg/ml at 20°C between 260–190 nm. Secondary structure contents were calculated according to Provencher and Glockner (1981).

In Vitro RNA Editing Assay

RNA editing-active protein extracts were prepared from mitochondrial vesicles isolated by nitrogen cavitation (Hauser et al., 1996). The vesicles were lysed as described by Göringer et al. (1994). Cleared extracts (~10 mg) were fractionated as in Pollard et al. (1992) and tested for their in vitro uridylate insertion RNA editing

activity using a precleaved editing assay. The three RNA reactants were prepared by solid-phase RNA synthesis: 5′ mRNA fragment (5′CL18): GGAAGUAUGAGACGUAGG; 3′ mRNA fragment (3′CL13): AUUGGAGUUAUAG-NH_2 (amino-modified at the 3′ end); gRNA (gPCA6-2A): CUAUAACUCCGAUAAACCUACGUCUCAUACUUCC. The 5′ mRNA fragment was radioactively labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. All radioactive RNA preparations were purified in urea-containing polyacrylamide gels and renatured in 6 mM HEPES/KOH (pH 7.5), 50 mM KCl, 2.1 mM $MgCl_2$, 0.1 mM Na_2EDTA, 0.5 mM DTT by heating to 70°C (2 min) followed by a slow cooling interval down to 30°C before chilling on ice.

Gene Silencing by RNAi

Gene silencing of *TbMP42* by RNAi was performed using the conditional RNAi system of Wang et al. (2000). A 701 bp DNA fragment from the 3′ end of *TbMP42* was cloned into plasmid pZJM. Ten micrograms of the plasmid was linearized with NotI and 10^9, cells of *T. brucei* strain 29-13 (Wirtz et al., 1999) were transfected by electroporation. Samples were transferred into 50 ml conditioned SDM 79 medium containing 20% (v/v) FCS, 50 μg/ml hygromycine (hyg), and 15 μg/ml neomycine (neo). After overnight incubation, phleomycine (2.5 μg/ml) was added, and antibiotic-resistant parasites were cultured for an additional 2 weeks. Clonal *TbMP42* RNAi cell lines were established by plating on agarose plates (Carruthers and Cross, 1992). The formation of TbMP42-specific dsRNA was induced by the addition of 1 μg/ml tetracyline (tet).

Analysis of the *TbMP42* RNAi Strain

Total RNA was isolated from 10^9 trypanosome cells according to Chomczynski and Sacchi (1987). Cells were harvested 24, 48, 72, and 96 hr after tet induction. The transcript abundance of *TbMP42* was measured by RT-PCR using the primers GGGTTTGTATTTG AAGACAAAGTTCTCC and AGAGGGTCCCTCGAAGTCTGTG. The abundance of TbMP42 after tet induction (48 hr) was verified by Western blotting using a monoclonal anti-TbMP42 antibody and a $(his)_6$-specific antibody. Poisoned primer extension reactions were performed as in Lambert et al. (1999).

Ribonuclease Activity Assays

RNA editing model substrates were prepared by solid phase RNA synthesis: U5 pre-mRNA: GGGAAAGUUUGUAUUUUUGCGAGUU AUAGCC; A5 pre-mRNA: GGGAAAGUUUGUAAAAAAGCGAGUU AUAGCC; gRNA: GGCUAUAACUCGCUCACAACUUUCCC. 50–250 pmol of the U5 or A5 pre-mRNAs was radioactively labeled either at their 5′ ends with T4 polynucleotide kinase and [γ-^{32}P]ATP (3000 Ci/mmol) or at their 3′ ends by T4-RNA ligase and (5′-^{32}P)-pCp (3000 Ci/mmol). Gel purification of the labeled pre-mRNAs was followed by annealing to the gRNA oligonucleotide, and the dsRNA product was further gel purified in semidenaturing (1 M urea) polyacrylamide gels. Annealed RNAs (50 fmol, specific activity 2–5 μCi/pmole) were incubated at 27°C for 3 hr with various concentrations of rTbMP42 in 20 mM HEPES/KOH (pH 7.5), 30 mM KCl, 10 mM $Mg(OAc)_2$, 5 mM $CaCl_2$, 1 mM $ZnSO_4$, 0.2 mM DTT, 0.5 mM ATP, 0.04 mM UTP. The cleavage products were separated in denaturing polyacrylamide gels and analyzed by phosphorimaging.

Optical Biosensor Measurements

The binding of rTbMP42 to different nucleic acid ligands was measured in real time using a resonant mirror system (Affinity Sensors). rTbMP42 (0.1 mg/ml in 20 mM $Na_xH_yPO_4$ [pH 7.4], 130 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$) was immobilized to the surface of a polyglutaraldehyde-activated amino silane microcuvette for 30 min at 27°C. Remaining activated sites were blocked with bovine serum albumin (BSA, 1 mg/ml) and washed. The rTbMP42-coated surface was equilibrated with binding buffer (20 mM HEPES [pH 7.8], 100 mM KCl, 1 mM $MgCl_2$, 1 mM $ZnCl_2$). Equilibrium dissociation constants (K_d) were derived from plots of the equilibrium resonant angle shifts as a function of the ligand concentration and fitted to the binding curve of the Langmuir adsorption isotherm. The following nucleic acid ligands were tested: ssRNA: CGGAUAUCAUACCGUC; dsRNA: GACGGUAUGAUAUCG/CGGA-UAUCAUACCGUC; ssDNA: GGATATACTATAACTCCA; dsDNA: TGGAGT-TATAGTATATCC/GGA TATACTATAACTCCA; DNA/RNA hybrid: TGGAGT-TATAGTATATCC/ GGAUAUACUAUAACUCCA.

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