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TbMP57 Is a 3′ Terminal Uridylyl Transferase (TUTase) of the *Trypanosoma brucei* Editosome

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Summary

RNA editing produces mature trypanosome mitochondrial mRNAs by uridylate (U) insertion and deletion. In insertion editing, Us are added to the pre-mRNA by a 3′ terminal uridylyl transferase (TUTase) activity. We report the identification of a TUTase activity that copurifies with in vitro editing and is catalyzed by the integral editosome protein TbMP57. TbMP57 catalyzes the addition of primarily a single U to single-stranded (ss) RNA and adds the number of Us specified by a guide RNA to insertion editing-like substrates. TbMP57 is distinct from a previously identified TUTase that adds many Us to ssRNA and which we find is neither a stable editosome component nor does it add Us to editing-like substrates. Recombinant TbMP57 specifically interacts with the editosome protein TbMP81, and this interaction enhances the TUTase activity. These results suggest that TbMP57 catalyzes U addition to pre-mRNA during editing.

Introduction

RNA editing in kinetoplastids transforms mitochondrial pre-mRNAs into translatable mature mRNAs by uridylate (U) insertion and deletion as specified by the sequence of small (∼60 nt) guide RNAs (gRNAs) (see Estevez and Simpson, 1999; Stuart and Panigrahi, 2002; Madison-Antenucci et al., 2002 for reviews). The central information regions of the gRNAs specify the editing sites and the number of Us to be added or removed. The 5′ regions of the gRNAs recognize their cognate pre-mRNAs by a complementary region of 4–14 nucleotides adjacent to the region to be edited and therefore function in cognate pre-mRNA and editing block selection. The gRNAs have posttranscriptionally added 3′ oligo (U) tails that have an unknown function, although they have been proposed to interact with the purine-rich regions of the pre-mRNAs upstream of the editing site (Blum and Simpson, 1990; Leung and Koslowsky, 1999). The U tail is dispensable in vitro (Burgess et al., 1999), but results reported here and elsewhere (Aphasizhev et al., 2002) imply that it may be essential in vivo.

The process of U insertion and deletion editing occurs by a coordinated series of enzymatic reactions. It begins with the formation of an “anchor” duplex between the gRNA and pre-mRNA. An endonuclease then cleaves the pre-mRNA, apparently at a mismatch between the pre-mRNA and the gRNA upstream of the anchor duplex, resulting in selection of the editing site. Us are then inserted by a 3′ terminal uridylyl transferase (TUTase) or removed by a U-specific exoribonuclease (ExoUase) subsequent to which, the RNA ends are ligated by an RNA ligase. Complete editing results in continuous base pairing between the gRNA and the pre-mRNA. Us are not only added to pre-mRNAs during insertion editing and to gRNA 3′ ends but also to the 3′ ends of mitochondrial rRNAs (Adler et al., 1991) and within the poly (A) tails of some mitochondrial mRNAs (Campbell et al., 1989).

Editing is catalyzed by a multiprotein complex that contains the endonuclease, TUTase, ExoUase and RNA ligase activities, and for simplicity, is called the editosome. Editosomes that contain these activities and catalyze in vitro editing have been purified from *T. brucei*. They sediment at ∼20S in glycerol gradients and have an estimated mass of ∼1600 kDa (Panigrahi et al., 2001a). Preparations of purified 20S editosomes have been reported to contain as few as 7 (Rusché et al., 1997) to as many as 13 (Madison-Antenucci et al., 1998) or 20 major proteins (Panigrahi et al., 2001a) depending on the isolation procedure, but accumulating data indicate that the larger number is more accurate. In *T. brucei*, TUTase activity sediments in glycerol gradients at ∼20S and at ∼35S–40S (Pollard et al., 1992; Corell et al., 1996). In addition, two chromatographically distinct TUTase activities sediment at ∼10S and ∼19S (McManus et al., 2000). Although these activities differed in the number of Us that they added to substrate RNAs, they were interpreted to be due to the same TUTase enzyme. Furthermore, multiple *Leishmania tarentolae* TUTase containing complexes with different RNA and protein components have been described (Peris et al., 1994; Byrne et al., 1995; Aphasizhev et al., 2002). Overall, these complexes may represent editosomes and other TUTase containing complexes in various stages of assembly, disassembly, and at different steps in editing or other processes.

Analyses in progress in several labs are characterizing various editosome components (see Stuart and Panigrahi, 2002; Madison-Antenucci et al., 2002 for reviews). To date, the identified editosome catalysts include two RNA ligases (McManus et al., 2001; Schnaufer et al., 2001; Huang et al., 2001; Panigrahi et al., 2001a) and a RNA helicase (Missel et al., 1997). A 3′ TUTase with a preprocessed molecular weight of 108 kDa has been identified in *T. brucei* and shown to be essential for editing and was thought to be the only TUTase in *T. brucei* mitochondria (Aphasizhev et al., 2002). However, most of this TUTase sediments at ∼10S, away from the bulk of editing activity, and does not appear to be a stable component of the editosome (Panigrahi et al., 2003). Since we are reporting the finding of another 3′ TUTase, we will refer throughout this paper to the 3′ TUTase identified by Aphasizhev et al. (2002) as TbTUTase 108. A potential editosome exonuclease, TbMP99, (for *T. brucei* mitochondrial protein of 99 kDa preprocessed) has been identified (A. Schnaufer et al., submitted), as

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have five related editosome proteins with RNase III or RNase III-related motifs, TbMP44, TbMP46, TbMP61, TbMP67, and TbMP90, that are candidate endonucleases (Panigrahi et al., 2003). Five other related editosome proteins, TbMP18, TbMP24, TbMP42, TbMP63, and TbMP81, contain oligonucleotide binding motifs (Panigrahi et al., 2001b, 2003; A. Schnaufer et al., submitted). TbMP81 and TbMP63 which also contain zinc fingers, appear to function in RNA and/or protein binding and perhaps coordination of the catalytic steps of editing (Huang et al., 2002; A. Schnaufer et al., submitted).

We report here that TbMP57 is a 3′ TUTase and a component of the editosome. TbMP57 cofractionates with in vitro editing activities and the ~1600 kDa, 20S editosome, but away from TbTUTase 108. The native editosomal TUTase activity catalyzes addition of one U to single-stranded (ss) RNA with a preference for a 3′ terminal A or G and adds the number of Us specified by gRNA to insertion editing-like substrates. TbMP57 specifically interacts with TbMP81, an essential component of the editosome (Drozdz et al., 2002), and its TUTase activity is enhanced by this interaction. In contrast, TbTUTase 108 elutes from Superose 6 columns at ~500 kDa, catalyzes the addition of many Us to ssRNA with no preference for the 3′ terminal nucleotide, and does not catalyze addition of the specified number of Us to editing-like substrates. These results indicate that TbTUTase 108 is not a stable component of the editosome and suggest that it has an alternate function in editing, perhaps addition of the U tail to gRNA. The results also indicate that TbMP57 is an integral component of the editosome and suggest that it adds the Us during insertion editing.

Results

Two TUTase Activities

TUTase activity was fractionated from cleared *T. brucei* mitochondrial lysate on two successive ion exchange columns followed by gel filtration on a Superose 6 column as previously described (Panigrahi et al., 2001a) (Figure 1). TUTase activity was measured by incorporation of radioactive UTP into yeast tRNA and by U addition to 5′-labeled 5′ CL18, a short ssRNA. Editing associated TUTase activity was monitored by a sensitive precleaved insertion editing assay that assesses gRNA-specified U addition and ligation with a double-stranded substrate consisting of 5′ and 3′ pre-mRNA fragments that mimic a pre-mRNA after cleavage and is therefore independent of endonuclease activity (see Figure 2, top panel) (see also Igo et al., 2000). Full-round deletion editing was monitored as previously described (data not shown) (Panigrahi et al., 2001a). TUTase activity assayed using the yeast tRNA substrate had a broad elution profile from the SP-Sepharose column (Figure 1A). We found that the peak of precleaved insertion and full-round deletion editing (fractions 9–19) contains a small peak of TUTase activity (fractions 12–14) while fractions 22–29 contain a larger peak of TUTase activity. Analyses using the 5′ CL18 substrate (Figure 1A) reveal that a multiple U addition activity (fractions 12–14) precedes but overlaps a single U addition activity (fractions 16–20) in the smaller TUTase peak. The single U addition activity trails into the larger peak of TUTase activity that primarily adds numerous Us. Not surprisingly, all fractions from this crude lysate have considerable nuclease activity as is evident from the bands that are smaller than the input RNA. Fractionation of the pooled SP-Sepharose fractions with editing activity (fractions 9–19) on a Q Sepharose column results in a single peak of TUTase activity, perhaps with a shoulder, that coelutes with editing activity (Figure 1B). Again a TUTase activity that adds numerous Us precedes and overlaps that which primarily adds a single U. Further separation of pooled fractions that contain the TUTase and editing activities (fractions 11–20) using a Superose 6 column results in two peaks of TUTase activity and a peak of activity which elutes in the void that has activity with yeast tRNA but not with 5′CL18 and was not studied further (Figure 1C). Peak I (fractions 19–24) has an apparent mass centered around 1600 kDa and coelutes with most of the precleaved insertion editing. This fraction also catalyzes full-round deletion and insertion editing (Panigrahi et al., 2001a; R.P.I., unpublished data). Most of the TUTase activity in this peak adds a single U to 5′CL18. Peak II (fractions 27–30) has an apparent mass centered around ~500 kDa, and most of the TUTase activity adds many Us to 5′CL18, again overlapping a single U addition activity. Thus, most of the activity that primarily adds a single U to ssRNA coelutes at ~1600 kDa with the editosome while most of the activity that adds many Us elutes at ~500 kDa.

Both TUTase Activities Are UTP Specific

The nucleotidyltransferase activities of the complexes in peaks I and II are essentially specific for UTP (Figure 2). Peak I TUTase activity primarily adds a single U to 5′CL18 at UTP concentrations between 1 and 1000 μM UTP, with substantial activity even at 1 μM UTP. Peak II TUTase activity primarily adds numerous Us between 10 and 1000 μM UTP, with more Us added with increasing UTP concentrations. Products with one or two added Us are observed throughout this concentration range with peak II. Both peaks add one or a few nucleotides from CTP but only at the higher concentrations, which might reflect small amounts of UTP in this commercial CTP or a low affinity of the enzyme(s) for CTP. Almost no addition is detected with ATP or GTP even at 100 and 1000 μM concentrations. The band above the input in the ATP lanes is due to circularization (ligation) of the input RNA. Precleaved insertion editing using 5′CL18 along with 3′CL13pp and gPCA6-2A gRNA, which specifies insertion of two Us and with 100 μM of each nucleotide, has a similar nucleotide specificity. Unlike with 5′CL18 alone, peak I TUTase adds primarily two Us to the precleaved insertion substrate in the presence of UTP, although some single Us were added. Edited RNA with two Us inserted (E2) is generated along with a trace of ligated 5′CL18 and 3′CL13pp without added Us (L) (Figure 2). A similar but less intense pattern is obtained with CTP, but the edited RNA obtained with CTP is less than 5% of that obtained with UTP. No addition products or edited RNA results with ATP or GTP, but ligated 5′CL18 and 3′CL13pp is produced, especially with ATP as previously observed (Igo et al., 2000). Like peak I, peak II has activity that primarily adds two Us to the precleaved editing substrate as well as ligation activity. However, there appears to be less of these activities

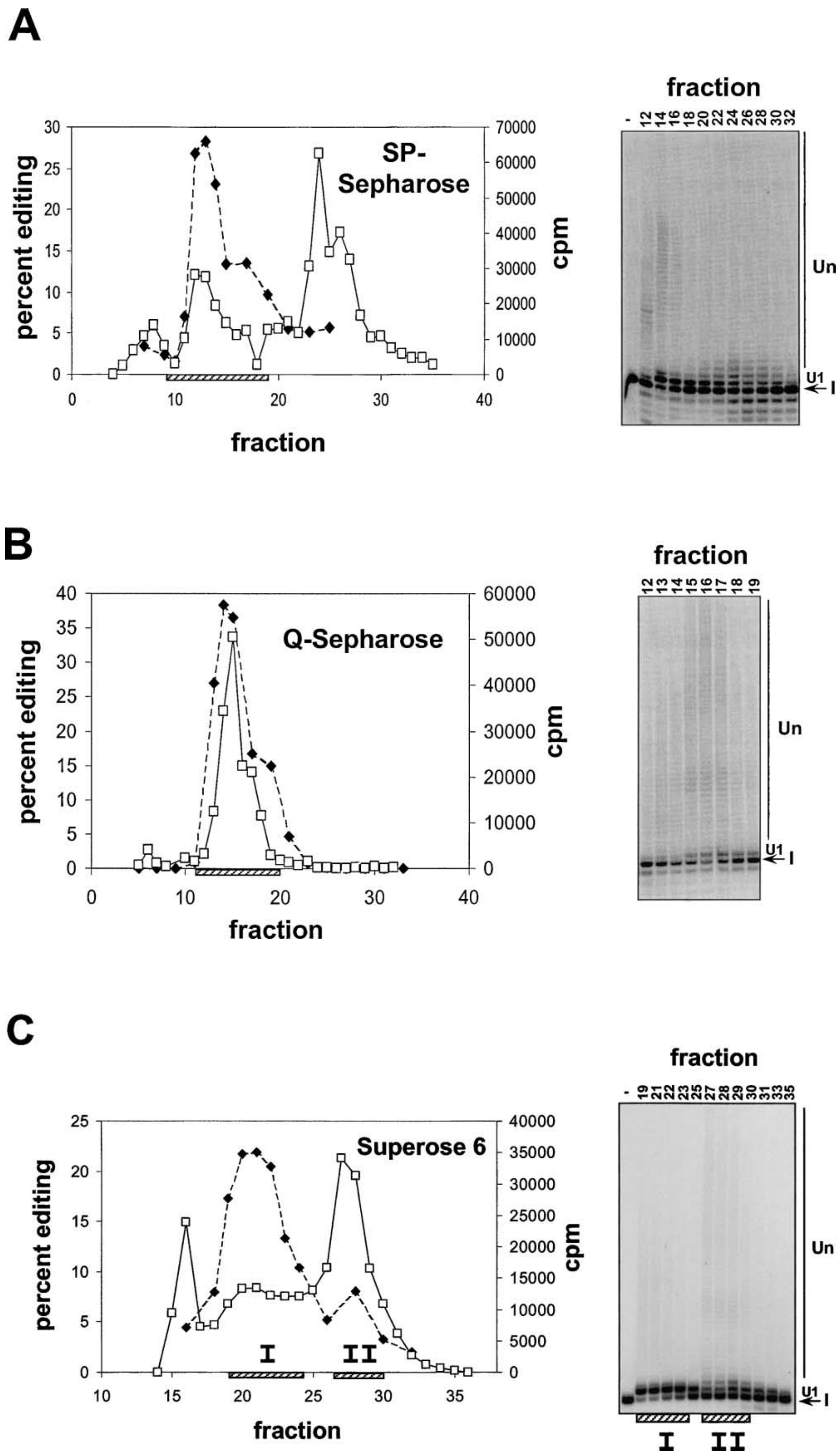

Figure 1. Fractionation of Mitochondrial TUTase Activities

Precleaved insertion editing activity (filled diamonds, dashed line) and TUTase activity with yeast tRNA substrate (open squares, solid line) in left panels, and TUTase activity with 5′CL18 substrate in right panels were assayed as described in the Experimental Procedures. Input RNA (arrow) and control with no added fraction (−) are indicated. Pooled fractions that were further purified are indicated by the crosshatched bars.

(A) Fractionation of cleared mitochondrial lysate with a SP Sepharose column.

(B) Fractionation of pooled SP Sepharose fractions 9–19 with a Q Sepharose column.

(C) Fractionation of pooled Q Sepharose fractions 11–20 with a Superose 6 column. Peaks I and II elute at ~1600 and ~500 kDa, respectively, compared to globular protein size standards.

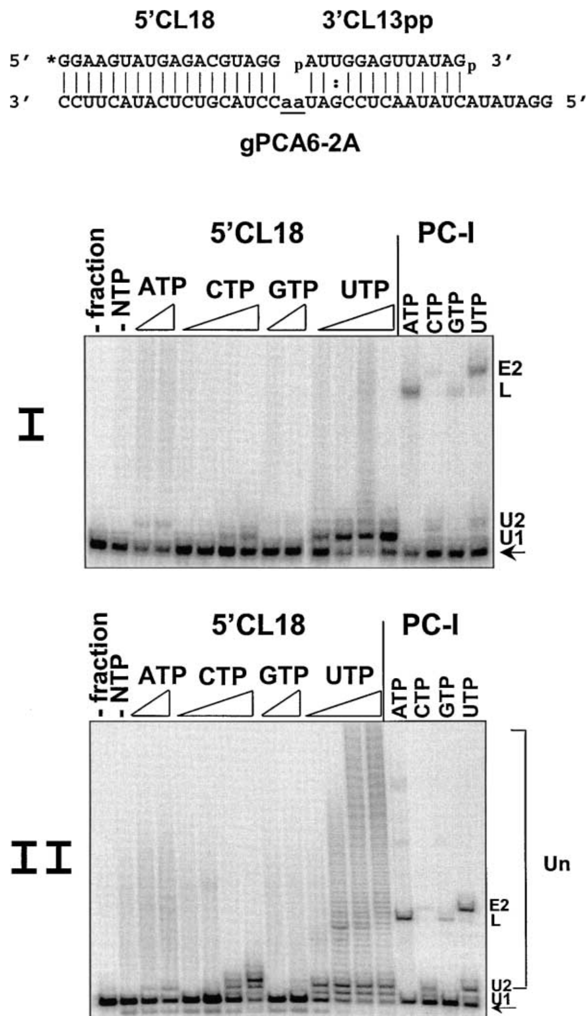

Figure 2. TUTase Activities Are U Specific

Substrate RNAs (top panel). 5′CL18 RNA was incubated with Superose 6 peak I (middle panel) or Superose 6 peak II (bottom panel) and with 1, 10, 100 μM, or 1 mM UTP or CTP or with 100 μM or 1 mM ATP or GTP to assay nucleotidyltransferase activity. 5′-labeled 5′CL18 in combination with 3′CL13pp and pPCA6-2A gRNA which specifies the insertion of two Us was incubated with Superose 6 peaks I or II and 100 μM of the indicated nucleotide to assay precleaved insertion editing activity (PC-I). See Experimental Procedures for details. The "− fraction" lanes contain 5′CL18 RNA (arrow) but no peak I or II material and "− NTP" lanes contain RNA and peak I or II material but no added nucleotide. Products with one (U1), two (U2), or more added Us (Un), ligated 5′CL18 and 3′CL13pp without added Us (L), and RNA edited by the insertion of two Us (E2) are indicated.

since peak II was concentrated (see Experimental Procedures) and contains a fragment or subcomplex of peak I material (Panigrahi et al., 2001b). Hence, the nucleotidyltransferase activities in peaks I and II are specific for UTP, and thus, both are 3′ TUTases.

The Two TUTase Activities Have Different 3′ Terminal Nucleotide Preferences

Peaks I and II have different preferences for the 3′ terminal nucleotide of the 5′CL18 substrate RNA. Peak I TUTase primarily adds one U to 5′CL18 with a 3′ terminal A or G, but there is little to no U addition with a 3′ terminal C or U (Figure 3A). The U-specific exonuclease (exoUase) in peak I removes the terminal U from the 3′ U substrate which could obscure U addition. However, substrates synthesized with *Sp*-phosphorothioate UTP that results in RNA in which the 3′ terminal U has an *Rp* linkage and is resistant to ExoUase (Igo et al., 2002b) produced the same results (data not shown). Peak II TUTase does not exhibit the strong terminal nucleotide preference seen with peak I but adds numerous Us to 5′CL18 with a 3′ terminal A, C, G, or U (Figure 3A). The reason for the variation in the number of Us added to substrates with different termini is uncertain, but the bands with one U added with A or G termini are probably due to the presence of a subcomplex of peak I editing complexes. The 3′ terminal nucleotide specificities were tested with precleaved editing substrates in which 5′CL18 (see Figure 2) had a 3′ A, C, G, or U and the corresponding base in the gRNA was changed to maintain base pairing (Figure 3B). Peaks I and II add two Us preferentially to a 3′ A or G, less so to a 3′ U, and even less so to a 3′C and produce edited RNA as is characteristic of editosomes (Igo et al., 2002a). Thus, peak II, which is concentrated, contains editosomes or fragments thereof. Hence, overall, the TUTase activities in peaks I and II differ in their terminal nucleotide preferences and the number of nucleotides they add to ssRNA.

TbMP57 Is a 3′ TUTase

TbMP57, an editosome protein that we previously identified (Panigrahi et al., 2003), is related to but distinct from the 108 kDa 3′ TUTase (TbTUTase 108) that was recently identified in *T. brucei* (and *Leishmania tarentolae*) and shown to be involved in RNA editing (Aphasizhev et al., 2002). The two different *T. brucei* proteins have 13% identity and 29% similarity over their full length. TbTUTase 108 contains additional N- and C-terminal sequences and has a zinc finger that is absent from TbMP57. The region of overlap has 24% identity and 57% total similarity by ClustalW. The sequences of both proteins predict mitochondrial localization signals. Both TUTases are predicted to contain nucleotidyltransferase domains (amino acids 63–144 in TbMP57) from PFAM database searches (http://www.sanger.ac.uk) and poly (A) polymerase core and associated domains (amino acids 278–329 and 365–424, respectively, in TbMP57) from PROSITE database searches (http://us.expasy.org) (Figure 4A). The catalytic signature of the nucleotidyltransferase domain places TbMP57 within the DNA polymerase β superfamily (Aravind and Koonin, 1999).

Recombinant TbMP57 specifically catalyzes U addition to ssRNA and precleaved insertion editing substrates (Figure 4B). Recombinant TbMP57 with an N-terminal Xpress tag that was transcribed and translated in vitro and immunoprecipitated with an anti-Xpress monoclonal antibody catalyzes nucleotide addition with radiolabeled UTP but not ATP, CTP, or GTP. Primarily a single U is added to 5′CL18 even when the UTP concentration is increased to 5 μM by addition of unlabeled UTP although 2, 3, and 4 Us were added to a lesser extent in some experiments (Figure 7B and data not shown). One and two Us are added to the precleaved editing substrate that specifies insertion of

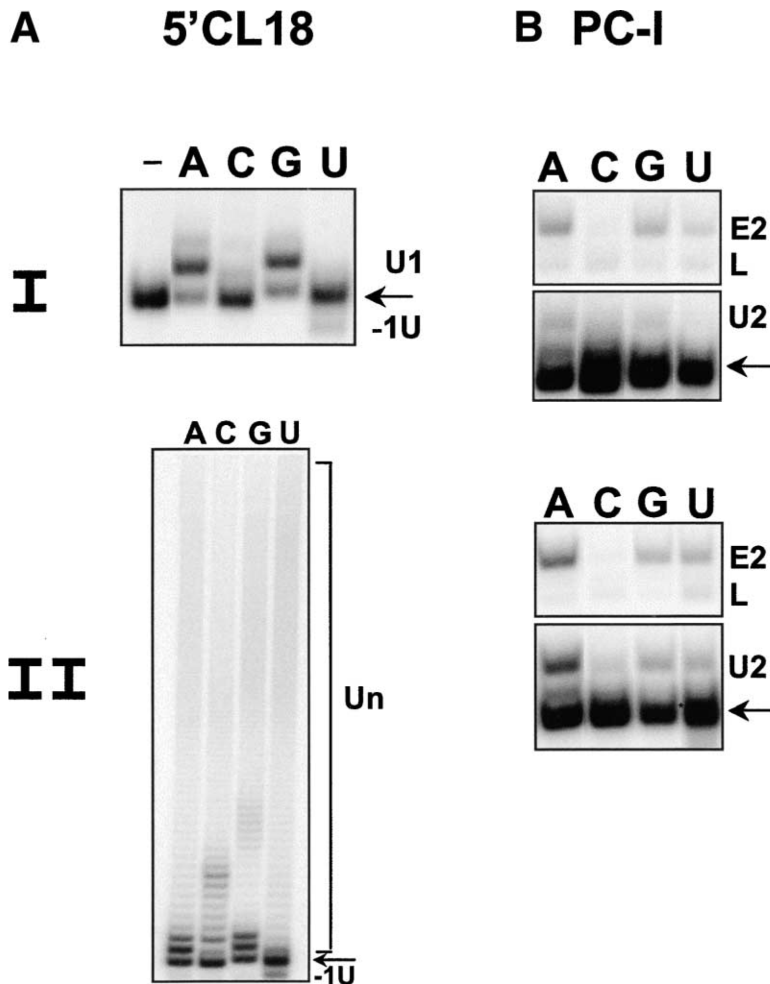

Figure 3. TUTases Differ in Substrate Terminal Nucleotide Specificities

(A) U addition by Superose 6 peak I or peak II to 5′ end-labeled 5′CL18 (arrow) with different 3′ terminal nucleotides. The 3′ terminal nucleotide is indicated above each lane and "− fraction" indicates a control in which fraction was omitted.

(B) Precleaved insertion editing (PC-I) by Superose 6 peak I or II. See Figure 1C for Superose 6 fractionations and Figure 2 for substrate details and other designations.

two Us (see Figure 2), and the proportion of product with two rather than one U is greater at higher UTP concentrations. These results indicate that TbMP57 is a 3′ TUTase.

The Two TUTases Are in Separate Complexes

Western analyses show that TbMP57 is primarily localized in Superose 6 peak I while TbTUTase 108 is primarily localized in peak II (Figure 5A). Polyclonal anti-TbMP57

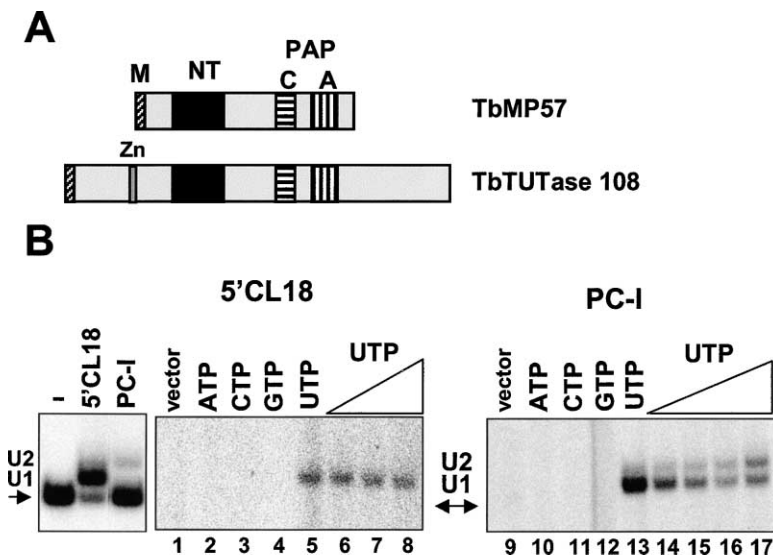

Figure 4. TbMP57 Is a 3′ TUTase

(A) Schematic of the alignment of TbMP57 and TbTUTase 108 using ClustalW (www.ebi.ac.uk). The nucleotidyltransferase (NT), poly (A) polymerase (PAP) core (C), and associated (A) domains and the mitochondrial targeting signal (M) that they share are indicated, as is the zinc finger (Zn) in TbTUTase 108.

(B) 3′ TUTase activity of recombinant TbMP57. N-terminal Xpress-tagged recombinant TbMP57 was immunoprecipitated with anti-Xpress monoclonal antibody and assayed for nucleotide addition to 5′CL18 or precleaved insertion editing substrates (PC-I) with 0.4 μM ^{32}P-labeled ATP, CTP, GTP, or UTP. Unlabeled UTP was added to the assays to a final concentration of 1 μM (lanes 6 and 14), 2.5 μM (lanes 7 and 15), 5 μM (lanes 8 and 16), and 10 μM UTP (lane 17). Vector lane contains the immunoprecipitate of reactions with vector without insert. Reactions using 5′ ^{32}P-labeled 5′CL18 and 100 μM UTP with Superose 6 peak I are shown for reference (left panel).

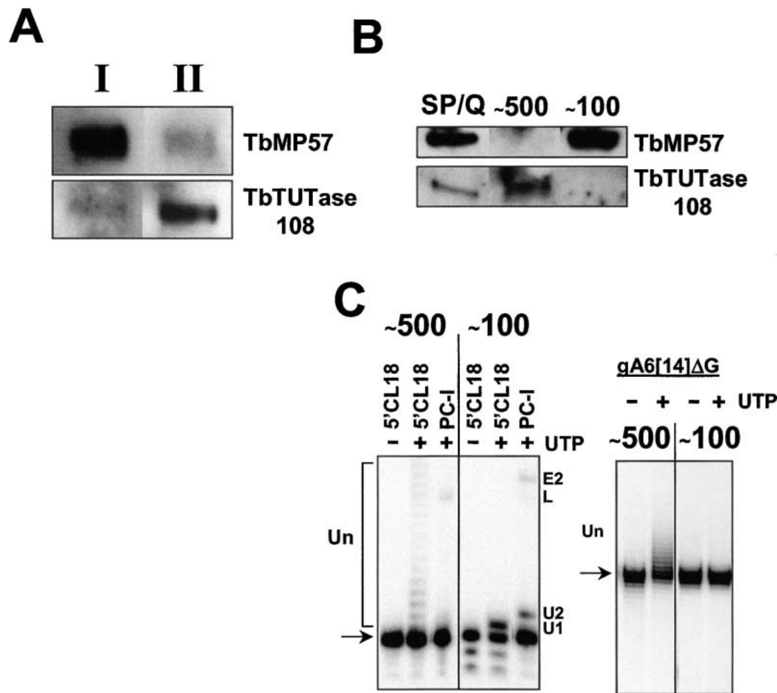

Figure 5. TbMP57 and TbTUTase 108 Are in Separate Complexes

(A) Western analysis of Superose 6 peaks I and II with affinity-purified polyclonal antisera specific for TbMP57 or TbTUTase 108.

(B) Western analysis of pooled Mono-Q column fractions 13–16 from Figure 1B (SP/Q) and TUTase peaks from Mono-Q columns that followed SP Sepharose and Superose 6 fractionation of mitochondrial lysates resulting in ~500 and ~100 kDa complexes (see Experimental Procedures for details) with anti-TbMP57 and TbTUTase 108 antisera.

(C) Assays of the TUTase peaks from a Mono-Q column (~500) and a Mono-Q column further purified on a Superdex-75 column (~100) with 5′ end-labeled 5′CL18 or the precleaved insertion substrate (PC-I) with or without UTP (left panel), or with 5′ end-labeled gA6[14]ΔG (right panel). See Experimental Procedures for details and Figure 2 for other designations.

antiserum produces substantial TbMP57 signal with Superose 6 peak I but little signal with peak II. Conversely, anti-TbTUTase108 antiserum (Aphasizhev et al., 2002) produces substantial TbTUTase 108 signal in peak II but little signal in peak I. Fractionation of pooled material from a SP Sepharose column (e.g., pooled fractions in Figure 1A) with Superose 6 before Mono-Q columns, as described in the Experimental Procedures, separates TbTUTase 108 from TbMP57 (Figure 5B). A peak of TUTase activity eluted from the Superose 6 column at ~500 kDa similar to peak II in Figure 1C but did not contain detectable precleaved insertion editing activity. Separation of the pooled active fractions on a Mono-Q column results in elution of a single peak of TUTase activity (data not shown) that contains TbTUTase 108 but not TbMP57 by Western analysis (Figure 5B). Also, the pooled peak fractions from this Mono-Q column catalyze the addition of numerous Us to 5′CL18 RNA, add numerous Us to gA6[14]ΔG gRNA, but do not catalyze precleaved insertion editing although some ligase activity is present (Figure 5C). Therefore, the presence of TbMP57 and precleaved insertion editing in Superose 6 peak II is probably a consequence of prior fractionation on the Q-Sepharose column (Panigrahi et al., 2001b).

A peak of TUTase activity that eluted from the Superose 6 column following the SP Sepharose column at ~100 kDa catalyzes precleaved insertion editing and predominately adds one U to 5′CL18. Fractionation of these pooled fractions with a Mono-Q column results in elution of two large (and two small) peaks of TUTase activity. Western analysis detected TbMP57 but not TbTUTase 108 in one of the large peaks and TbTUTase 108 but not TbMP57 in the other (Figure 5B and data not shown). Thus, TbMP57 and TbTUTase 108 exist in separable complexes. Fractionation of the pooled TbMP57 fractions with a Superdex-75 column results in elution of a single peak of TUTase activity at ~100 kDa. This peak contains precleaved insertion editing and TUTase activity that adds one U to 5′CL18 but does not add Us to gA6[14]ΔG gRNA (Figure 5C). This peak may represent a fragment of the larger TbMP57 containing complex.

TbMP57 Is an Integral Component of the Editosome

Editosomes were immunoprecipitated from pooled fractions (13–16) from Mono-Q columns that contain both TbMP57 and TbTUTase 108 (Figures 1B and 5A) using a monoclonal antibody that is specific for the TbMP63 editosome component and immunoprecipitates editosomes that are active in in vitro editing (Panigrahi et al., 2001b). Western analysis detected TbMP57 but not TbTUTase 108 in the immunoprecipitates (Figure 6A). The immunoprecipitates primarily catalyze addition of a single U to 5′CL18 (Figure 6B) with a preference for RNA with a 3′ terminal A or G (data not shown). The anti-TbMP63 precipitates also contain most of the editing activity although some remains in the supernatant (Figure 6C). Similar results were obtained with immunoprecipitates prepared from crude mitochondrial lysate or ~20S glycerol gradient fractions of such lysates (data not shown). The activity that adds numerous Us to 5′CL18 remains in the anti-TbMP63 supernatant (Figure 6B). This supernatant has reduced editing activity but contains substantial activity that adds two Us to precleaved editing substrates (Figure 6C). The latter retained activity may be due to residual TbMP57 and appear enhanced because of reduced ligase activity or less likely may be due to TbTUTase 108. Immunoprecipitates made with anti-TbTUTase 108 antiserum contain the activity that adds numerous Us to 5′CL18 as seen by the smear of products with apparently hundreds of added Us at the top of the gel while the activity that

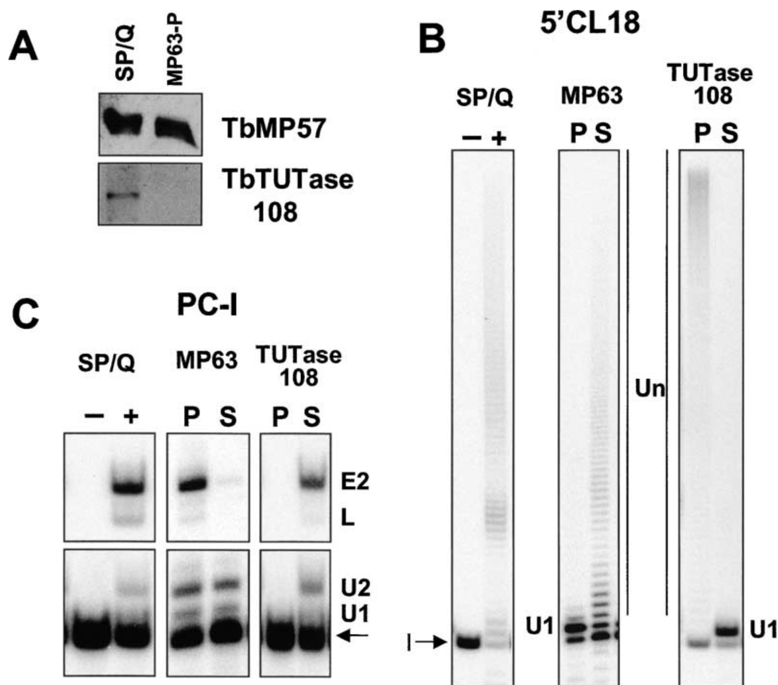

Figure 6. Immunoseparation of TbMP57 and TbTUTase 108 Complexes

(A) Western analysis of pooled SP/Q fractions 13–16 as in Figure 1B and the anti-TbMP63 immunoprecipitate using anti-TbMP57 and anti-TbTUTase 108 antisera. TUTase analyses with (B) 5′CL18 and (C) precleaved insertion (PC-I) substrates of precipitates (P) and supernatants (S) from immunoprecipitations of the pooled SP/Q fractions using anti-TbMP63 monoclonal antibody (Panigrahi et al., 2001b) or polyclonal anti-TbTUTase 108 antiserum (Aphasizhev et al., 2002). Assays and other designations are as in Figure 2.

adds a single U remains in the supernatant (Figure 6B). The supernatant catalyzes precleaved insertion editing, while the immunoprecipitate does not (Figure 6C). The lack of precleaved insertion editing activity in the anti-TbTUTase 108 precipitate suggests that the U addition activity in the anti-TbMP63 supernatant (Figure 6C, middle panel) is due to residual TbMP57. These results along with those in Figure 5 show that TbMP57 and TbTUTase 108 are in separable complexes and that TbMP57 is an integral component of the editing complex that adds Us during in vitro insertion editing while TbTUTase 108 is not stably associated with this complex.

TbMP81 Interacts with TbMP57 and Enhances Its Activity

The interaction of TbMP57 with other editosome proteins was investigated by coimmunoprecipitation of ^{35}S-labeled recombinant proteins with specific antibodies. Immunoprecipitation of in vitro transcribed and translated recombinant TbMP57 using a monoclonal antibody to its N-terminal Xpress tag coprecipitates TbMP81, which is an essential component of the editosome with a role in insertion editing (Drozdz et al., 2002) (Figure 7A). The Xpress tag antibody immunoprecipitates TbMP57 alone but not TbMP81 in the absence of TbMP57. Similarly, immunoprecipitation of TbMP81 using an anti-TbMP81 monoclonal antibody (Panigrahi et al., 2001b) coprecipitates TbMP57 (Figure 7A). The anti-TbMP81 antibody immunoprecipitates TbMP81 alone but not TbMP57 in the absence of TbMP81. No coimmunoprecipitations were detected between TbMP57 and TbMP24, TbMP42, TbMP44, TbMP63, TbREL1, or TbREL2 editosome proteins (data not shown). The interaction with TbMP81 enhances the activity of TbMP57. Immunoprecipitated recombinant TbMP57 primarily adds one U but also adds multiple Us to 5′CL18 and primarily adds two Us to a precleaved substrate (Figure 7B). While TbMP81 precipitates have no TUTase activity, the TUTase activity of TbMP57 is increased 4-fold in coprecipitates made with antibodies specific for either TbMP57 or TbMP81 (Figure 7B). Thus, not only is TbMP57 an integral component of the editosome but its activity is enhanced by another component of the editosome.

Discussion

We report here that TbMP57 is a 3′ TUTase that is a stable component of the multiprotein complex that catalyzes RNA editing in *Trypanosoma brucei*. TbMP57 is related to TbTUTase 108 that is essential for editing (Aphasizhev et al., 2002), but these TUTases occur in different complexes that were separated by biochemical and immunological methods. Purified native complexes that contain TbMP57 predominately add one U to ssRNA with a preference for a 3′ terminal A or G. They also add the number of Us specified by gRNA to an editing substrate with a bias against addition to a 3′ terminal C. In contrast, native complexes that contain TbTUTase 108 add numerous Us to ssRNA with no 3′ terminal nucleotide preference. Recombinant TbMP57 coprecipitates with TbMP81, an essential protein of the editosome and TUTase activity is enhanced in the coprecipitates. We thus designate TbTUTase 108 as *T. brucei* RNA editing TUTase 1, TbRET1, and TbMP57 as TbRET2. These results show that TbRET2 is an integral component of the editing complex and indicate that it's likely function is to catalyze the U additions during insertion editing. They also show that TbRET1 is associated with a different complex, which suggests that it may function in addition of the 3′ U tails to gRNAs.

TbRET2 is stably associated with the complex that

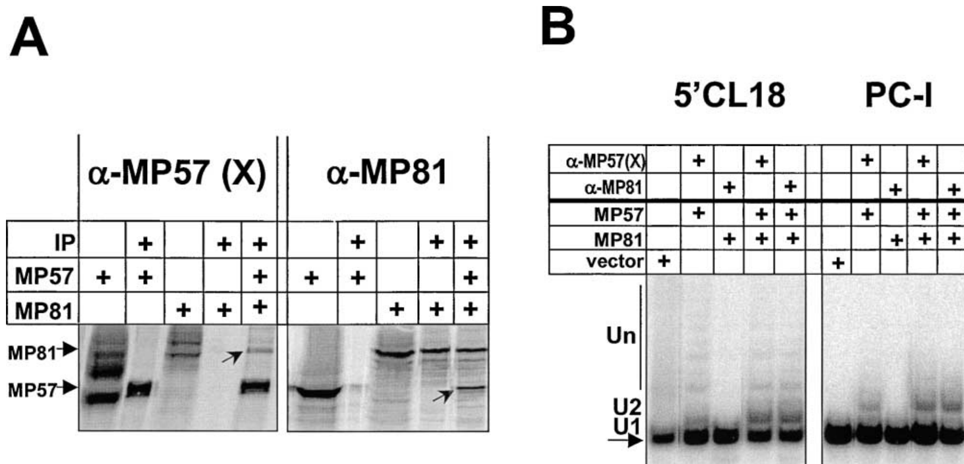

Figure 7. TbMP57 Interacts with TbMP81

TbMP57 with an N-terminal Xpress tag and TbMP81 were expressed by in vitro transcription/translation as described in the Experimental Procedures.

(A) Coprecipitation of TbMP57 and TbMP81. ^{35}S-labeled products were separated on polyacrylamide gels directly or after immunoprecipitation with anti-Xpress Mab (Invitrogen) for TbMP57 (α-MP57 [X]) or anti-TbMP81 MAb (Panigrahi et al., 2001b). The arrows indicate the coprecipitated proteins.

(B) Assays of TUTase activity as in Figure 3 using unlabeled precipitates prepared as in (A). Vector lane contains the precipitate of reactions with vector without insert. Input RNA (arrow), and U1, U2, and Un addition products are indicated.

catalyzes RNA editing. It copurifies with this complex by biochemical methods, by immunoprecipitation with monoclonal antibodies specific for editosome components and by TAP-tag purification of an editosome protein (Panigrahi et al., 2003). These purified complexes contain the endonuclease, ExoUase, TUTase, and RNA ligase editing activities as well as full-round in vitro editing. In addition, TbRET2 interacts with TbMP81, an essential editosome protein (Drozdz et al., 2002). This interaction is evident from coimmunoprecipitation of recombinant TbRET2 with TbMP81 and the consequent enhancement of TbRET2 TUTase activity (see Figure 7), yeast two-hybrid analysis, and the presence of the two proteins in a TAP-tagged editing subcomplex (A. Schnaufer et al., submitted). Importantly, repression of TbMP81 expression by dsRNAi in both procyclic and bloodstream form *T. brucei* results in the preferential loss of insertion editing (Drozdz et al., 2002), indicating a role for TbMP57 in insertion editing. In contrast to TbRET2, TbRET1 does not appear to be stably associated with the complex that contains the set of catalytic activities for editing. It is not detected by tandem mass spectrometry in complexes that catalyze in vitro editing purified by any of the aforementioned methods (Panigrahi et al., 2003). It is also found in a different complex that elutes from a Superose 6 column after two ion exchange columns at ~500 kDa (Figure 5). The composition of the ~500 kDa TbRET1 complex is unknown but may be a homomultimer since the recombinant *L. tarentolae* homolog of TbRET1 (LtRET1) was shown to multimerize resulting in a complex of ~500 kDa in vitro which was similarly observed in vivo and has similar characteristics to Superose 6 peak II (Figures 2 and 3) (Aphasizhev et al., 2002). The ligase activity in the ~500 kDa TbRET1 complex after elution from a Mono-Q column (Figure 5), may represent adventitious association or a functional association, perhaps via RNA binding. Similarly a minor fraction of ligase protein was coimmunoprecipitated with LtRET1 (Aphasizhev et al., 2002). Overall, the two TUTases are primarily in distinct complexes. TbRET2 is in the ~1600 kDa editosomal complex that catalyzes in vitro editing (Panigrahi et al., 2003). It also occurs in a ~100 kDa complex that may be related to a recently observed tripartite subcomplex composed of TbRET2, TbMP81, and an RNA editing ligase, TbREL2 (A. Schnaufer et al., submitted).

The TbRET2 complex catalyzes in vitro precleaved insertion editing, while the TbRET1 complex does not. The simplest possibility is that TbRET2 catalyzes U addition to pre-mRNAs during insertion editing and that TbRET1 adds the 3′ U tail to gRNAs as implied by the two separable complexes. The catalytic characteristics of the enzymes are also consistent with this possibility. TbMP57 complexes preferentially add U to ssRNA ending in A or G and precleaved editing substrates ending in A, G, or U (Figure 3). These preferences reflect the characteristics of natural insertion editing sites which have a predominance of terminal A and G, a low level of C, and absence of Us (Burgess and Stuart, 2000). Although TbMP57 complexes add Us to a 3′ terminal U, their absence in natural editing sites is simply the consequence of base pairing of the U with the guiding A or G in the gRNA and therefore lack of cleavage at that site. The bias against C may be due to substrate or nucleotide selectivity and/or reflect evolutionary selection against potential base pairing of C with a guiding G in gRNA. The addition of numerous Us to RNAs ending in any nucleotide by TbRET1 (Figure 3) (Aphasizhev et al., 2002) parallels the presence of any 3′ nucleotide in the cellular gRNA population upstream of the U tail. In addition, immunoprecipitates of LtRET1 contain ~40% of the cellular gRNAs (Aphasizhev et al., 2002). If these

separate functions for the two TUTases are the case, then, since TbRET1 is essential for editing (Aphasizhev et al., 2002), to our knowledge this would be the first evidence that the gRNA 3′ U tails are essential for editing. Nonencoded uridylates are also added to the 3′ ends of ribosomal RNAs (Adler et al., 1991) and within the poly (A) tails of pre-mRNAs (Campbell et al., 1989) in the mitochondrion. It is unclear which TUTase, if either, functions in these additions.

Native TbRET2 primarily adds a single U to ssRNA and the specified number of Us to a precleaved editing substrate (Figure 2). Recombinant TbRET2 adds a single U to substrates at low UTP concentrations (Figure 4) but is capable of adding more than one U to ssRNA, especially at higher UTP concentrations (Figure 7 and data not shown). The reason for this difference may be that the activity of TbRET2 is altered in native complexes by its association with other proteins (e.g., TbMP81) as well as its conformation in the editing complex or possibly by posttranslational modifications. Recombinant and native RET1 adds numerous Us to ssRNA (Aphasizhev et al., 2002; and this study). Recombinant LtRET1 adds Us to double-stranded RNA but native TbRET1 complexes in this study did not, possibly because of its association with other proteins. If TbRET1 adds gRNA U tails which average ~14 nt, the length must be controlled perhaps by a U specific endo- or exonuclease (McManus et al., 2000) which might be associated with the TbRET1 complex.

The presence of the two *T. brucei* TUTases in different complexes does not negate the possibility that the complexes may interact. There are trace amounts of TbRET1 in the Superose 6 peak I fraction and trace amounts of TbRET2 in the peak II fraction. This might represent a functional interaction or be due to cross contamination. The latter is implied by the separate immunoprecipitation of the complexes, although weak interactions might be disrupted during immunoprecipitation. In *L. tarentolae*, the glycerol gradient sedimentation profiles of high molecular weight LtRET1 and ligase-containing complexes (that contain the homolog of TbMP57) overlap but the sedimentation of the LtRET1 complex shifts with micrococcal nuclease treatment (Aphasizhev et al., 2003). This resembles the micrococcal nuclease sensitive immunoprecipitation of in vitro editing activity with a monoclonal antibody specific for gBP21, a protein that can bind gRNA (Allen et al., 1998). Thus, the TbRET2 and TbRET1 complexes may physically interact, possibly via RNA. Whether or not the two TUTase complexes physically interact, a functional interaction in editing is implied by the loss of editing upon knockdown of TbRET1 gene expression (Aphasizhev et al., 2002). This might represent the requirement for gRNA 3′ U tail addition, but it cannot yet be excluded that TbRET1 may add Us to pre-mRNA during editing, perhaps during transient association with the TbMP57 complex. This seems unlikely, as discussed above, but the functions may not be mutually exclusive much as the two editing ligases do not have mutually exclusive functions in insertion versus deletion editing (Drozdz et al., 2002).

Recombinant TbRET2 is a catalytically active TUTase, consistent with its nucleotidyltransferase and core and associated poly (A) polymerase motifs. The 4-fold stimulation of TbRET2 activity in the presence of associated TbMP81 is intriguing. Database searches reveal that the C-terminal region of TbRET2 that contains the PAP core and associated domains is related to a group of proteins in the DNA polymerase β superfamily that includes recently identified cytoplasmic poly (A) polymerases (Keller and Martin, 2002). Such proteins, including TbRET2, contain the nucleotidyltransferase and central domains similar to classical poly (A) polymerases but lack an identifiable RNA recognition motif (RRM)-like domain. The *C. elegans* GLD-2 protein is the prototype for this family of regulatory cytoplasmic poly (A) polymerases, the hallmark of which is that they function as heterodimers (Wang et al., 2002). GLD-3 is the binding partner of GLD-2 and likely mediates, at least in part, RNA binding and substrate specificity. GLD-2 has low polymerase activity on its own but robust activity in the presence of GLD-3. The C-terminal domain of GLD-2 is essential for interaction with GLD-3. By analogy, we propose that TbMP81, which has an oligonucleotide binding fold (OB-fold) motif (A. Schnaufer et al., submitted) and zinc fingers, functions much like GLD-3 by mediating RNA binding and substrate specificity for TbRET2.

As discussed elsewhere, TbMP81 also interacts with the RNA editing ligase, TbREL2 (A. Schnaufer et al., submitted). Indeed this study showed that knockins of TAP-tagged TbREL2 results in tagged editosomes that are 20S, as is normal, as well as ~8S subcomplexes that are composed of the tagged ligase, TbMP81, and TbRET2. These subcomplexes catalyze precleaved insertion editing. Hence, TbMP81 was proposed to coordinate the sequence of events in insertion editing. Similarly, TbMP63, which is related to TbMP81, interacts with the other editing ligase, TbREL1, and a potential exoUase, TbMP99, and has been proposed to coordinate the events in deletion editing (A. Schnaufer et al. submitted).

It is possible that multiple complexes, including a core catalytic complex (with the endonuclease, TbRET2, ExoUase, TbREL1, and TbREL2 catalysts), a TbRET1 complex that may add gRNA 3′ U tails, and perhaps other complexes and soluble protein factors dynamically interact during the process of editing.

Experimental Procedures

Cell Growth and Mitochondria Isolation

T. brucei procyclic forms (strain IsTaR 1.7a) were grown to log phase in vitro as previously described (Stuart et al, 1984). The mitochondrial vesicles were isolated as previously described (Harris et al., 1990) and stored at −70°C.

Fractionation of TUTase Activities

TUTase activities were fractionated by a combination of ion exchange and gel filtration chromatography according to Panigrahi et al. (2001a). Briefly, cleared mitochondrial lysate was fractionated with a SP Sepharose HR cation exchange column (Pharmacia, Piscataway, NJ) and fractions (9–19) that had both in vitro editing and TUTase activities were pooled and fractionated on a Q Sepharose column (Pharmacia). Fractions (11–20) that were positive for editing and TUTase activities were pooled, concentrated, and size fractionated using a Superose 6 HR (10/30) column (Pharmacia). A variation of this procedure reversed the order of the Superose-6 and Mono-Q columns. Cleared mitochondrial lysate (148 mg protein) was loaded onto a 5 ml SP Sepharose column and eluted into 2 ml fractions at a 1 ml/min flow rate with a 40 ml linear 50–330 mM KCl gradient followed by 40 ml linear gradient to 1 M KCl. Fractions 9–20 that

contained all editing and some TUTase activity (similar to Figure 1A) were pooled, concentrated, and fractionated with a Superose 6 HR (10/30) gel filtration column (Pharmacia). Fractions 27–30 that contained TUTase activity but no editing activity were pooled and loaded onto 1 ml Mono-Q HR 5/5 anion exchange column and eluted at a 0.5 ml/min flow rate into 500 μl fractions with a 6 ml linear gradient to 330 mM KCl followed by a 6 ml linear gradient to 1 M KCl. Alternately, cleared mitochondrial lysate (from 1.6×10^{12} cells) was loaded onto a 25 ml SP Sepharose column in the same buffer as above and eluted in 4 ml fractions at a 1 ml/ min flow rate with a 300 ml linear salt gradient of 50 mM to 1 M KCl. Fractions 18–31 were loaded onto a Superose-6 HR 10/30 size exclusion column as above. Fractions 61–70 were loaded onto a Mono-Q HR 5/5 anion exchange column (Pharmacia) at 1 ml/min flow rate, and the bound proteins were eluted with an 8 ml linear salt gradient of 50–330 mM KCl. The TUTase peak in fractions 7–14 was fractionated on a Superdex-75 column as previously described (Salavati et al, 2002). Sizes were estimated by comparison with elution of globular protein size standards (Gel filtration HMW Calibration Kit; Pharmacia) from the Superose 6 columns.

Preparation and Labeling of RNAs

RNAs were transcribed using T7 RNA polymerase (Promega, Madison, WI) from PCR-generated templates. 3′CL13pp was purchased as an oligoribonucleotide from Oligos, Etc. 5′CL18 and variants with different 3′ termini as well as the guide RNAs used in precleaved editing assays were prepared as described (Igo et al., 2000). 5′CL18 RNA and its derivatives were labeled at the 5′ terminus by phosphorylation of alkaline phosphatase treated RNA with T4 polynucleotide kinase (GIBCO-BRL, Carlsbad, CA) in the presence of [γ-^{32}P]-ATP (Dupont NEN, Wilmington, DE). gA6[14]Δ16G was prepared as described (Seiwert and Stuart, 1994) and 5′ labeled by capping with guanylyltransferase (GIBCO-BRL) in the presence of [γ-^{32}P]-GTP (Dupont NEN).

TUTase Assays

TUTase activity was assayed essentially as described (Bakalara et al., 1989; Pollard et al., 1992; Corell et al., 1996). 1 μl of each ion exchange column fraction or 5 μl of each Superose 6 column fraction was incubated with 1 μg yeast tRNA in the presence of 5 μCi [α-^{32}P]-UTP (800 Ci/mmol) (Dupont NEN) for 1 hr at 27°C in buffer containing 25 mM HEPES, pH 7.9, 10 mM magnesium acetate, 0.5 mM dithiothreitol, and 1 mM EDTA. For ion exchange column fractions, KCl was added to a final concentration of 50 mM. One-fourth of the reaction mixture (5 μl) was spotted onto Whatman GF/C microfiber discs, and the labeled products were precipitated with 10% TCA with 100 mM sodium pyrophosphate at 4°C. Incorporated radioactivity was measured by liquid scintillation counting. U addition to 5′CL18 (0.1–0.2 pmol) or gA6[14]ΔG (0.25 pmol) was performed in 30 μl reaction volumes containing 25 mM HEPES, pH 7.9, 10 mM magnesium acetate, 5 mM calcium chloride, 0.5 mM dithiothreitol, and 1 mM EDTA and 3–5 μl each column fraction. UTP was added to a final concentration of 100 μM unless otherwise specified. For analysis of the TUTase activity in Superose 6 peak I, fractions 19–24 were pooled, and 3 μl was used in each reaction. For analysis of the TUTase activity in Superose 6 peak II, fractions 27–30 were pooled and concentrated between 4- to 8-fold, and 3 μl was used in each reaction. Reaction products were phenol-chloroform extracted, ethanol precipitated, and run on 11% polyacrylamide-7 M urea gels and visualized by phosphorimaging (Storm PhosphorImager; Molecular Dynamics).

Precleaved Insertion Editing Assays

Precleaved insertion editing assays were performed as previously described (Igo et al., 2000). Briefly, editing reactions containing labeled 5′CL18, 3′CL13pp, and gPCA6-2A were incubated with 3–5 μl of individual column fractions, 3 μl of Superose 6 peak I, or 3 μl of 4×–8× concentrated Superose 6 peak II for 3 hr at 27°C in the presence of 100 μM UTP or other nucleotides unless otherwise specified. The reaction products were phenol-chloroform extracted, ethanol precipitated, and run on 11% polyacrylamide-7 M urea gels and visualized by phosphorimaging.

Cloning, Expression, and Purification of TbMP57

Mass spectrometric analysis of purified editosomes identified genomic DNA sequences 9C2tf that contained the start codon of TbMP57, and 9C14tr that contained a portion of the 3′ end of the TbMP57 gene (http://www.tigr.org) (Panigrahi et al., 2003). The complete ORF of TbMP57 was obtained by a combination of PCR and RT-PCR. The full-length ORF minus the predicted mitochondrial localization signal was amplified from genomic DNA with primers 4017 (ATTA*TCTCGAG*TCGCCGCTGTCGCTCCCCAGTAC) and 4018 (TAC*AAGCTT*TCATTCACGGTCTCTGTCACGC) and cloned into pRSET A (Invitrogen). The restriction sites are in italics. BL21 DE3-pLysS cells were transformed with the resultant plasmid, and protein expression was induced with 0.4 mM IPTG at 37°C for 2–3 hr. Recombinant TbMP57 was mostly insoluble with this procedure and was therefore purified by its N-terminal 6×His tag on a ProBond nickel resin column using denaturing conditions as per the manufacturers instructions (Novagen).

Polyclonal Antibodies and Western Analyses

Rabbit polyclonal antiserum was generated to purified recombinant TbMP57 by Pocono Research Farm and Laboratories. The antiserum was affinity purified by absorption to recombinant protein immobilized on PVDF membrane followed by elution similar to published procedures (Olmsted, 1981). Bacterial lysate containing recombinant TbMP57 was separated by SDS-PAGE, transferred to PVDF membrane (Millipore, Bedford, MA), and stained with Ponceau S. A strip containing the recombinant protein was cut from the membrane and blocked in 5% milk in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween). TbMP57 antiserum (1 ml) was diluted 1:5 in TBST containing 0.1% BSA and incubated with the antigen strip overnight at room temperature. The strip was washed three times in TBST. The bound antibodies were eluted with buffer containing 0.1 M glycine, pH 2.8, 500 mM NaCl, and 0.05% Tween 20, neutralized with 0.3 vol of 1 M Tris, pH 8.1, and stored with 1.5% BSA. TbTUTase 108 rabbit polyclonal antiserum was a kind gift from Larry Simpson (Aphasizhev et al., 2002). The antiserum (100 μl) was affinity purified by the same procedure using immobilized recombinant TbTUTase 108 that was expressed as previously described (Aphasizhev et al., 2002). For immunoblot analysis, proteins were separated by SDS-PAGE, transferred to PVDF membranes, and probed with 1:10 dilutions of the affinity-purified polyclonal antisera.

Cell-free Protein Expression and Coimmunoprecipitation of Recombinant Proteins

pRSET-MP57 with an N-terminal Xpress epitope tag (Invitrogen) and pSG-MP81 (A. Schnaufer et al., submitted) were used for expression of recombinant proteins. Recombinant proteins were expressed individually using the TNT T7 Quick Coupled Transcription/Translation System (Promega) in the presence of unlabeled or ^{35}S- labeled methionine as per the manufacturer's instructions. Mixtures of the proteins were preincubated on ice for 5 min. TbMP57 and TbMP81 were immunoprecipitated with anti-Xpress (Invitrogen) and anti-TbMP81 (P4D8) monoclonal antibodies (Panigrahi et al., 2001b), respectively. Immunomagnetic beads (0.5–1 $\times$ 10^8 beads) (Dynabeads M-450; Dynal, Lake Success, NY) coated with goat anti-mouse IgG were coupled with 2.5 μl anti-Xpress antibody in IP buffer (10 mM Tris-HCl, pH 7.2, 10 mM $MgCl_2$, 200 mM KCl, and 0.1% Triton X-100) or 1 ml P4D8 tissue culture supernatant in the presence of 1% BSA for 90 min at 4°C with mixing. The beads were washed three times with 800 μl IP buffer and incubated with either individual proteins or mixtures of proteins in IP buffer containing 1% BSA for 90 min at 4°C with mixing. The beads were washed four times with 800 μl IP buffer. ^{35}S-labeled proteins were run out on 10% SDS-polyacrylamide gels, dried, and visualized by phosphorimaging. Unlabeled immunoprecipitated proteins were assayed for U addition to 5′CL18 and precleaved insertion editing substrates as described above except with the omission of torula RNA. Assays with radiolabeled nucleotides contained 200 fmol unlabeled 5′CL18 and 10 μCi α-^{32}P-NTP (800 Ci/mmol) for a final concentration of 0.4 μM of each nucleotide. For higher UTP concentrations, unlabeled UTP was added to a final total UTP concentration of 1, 2.5, 5, or 10 μM.

Immunoprecipitations

Immunoprecipitations were performed as previously described with some modifications (Panigrahi et al., 2001a). Goat anti-mouse IgG-

coupled immunomagnetic beads (5×10^7 beads) (Dynabeads M-450; Dynal) were coated with anti-TbMP63 monoclonal antibody (P1H3-D7) (Panigrahi et al., 2001b) from 1 ml tissue culture supernatant in the presence of 1% BSA at 4°C and mixed for 90 min. Alternatively, sheep anti-rabbit IgG-coupled beads (6×10^7 beads) (Dynabeads M-280; Dynal) were coated with 100 μl affinity-purified TbTUTase 108 antiserum (Aphasizhev et al., 2002) overnight at 4°C. The beads were washed three times with IP buffer. The antibody-bound beads were incubated with 40 μl SP/Q pooled fractions 13–16 (Figure 1B) in 1× IP buffer and 1% BSA for 90 min at 4°C and mixed. The beads were washed four times with IP buffer and assayed for uridylate addition to 5′CL18 and precleaved insertion editing as described above.

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Accession Numbers

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