Separate Insertion and Deletion Subcomplexes of the *Trypanosoma brucei* RNA Editing Complex

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Summary

The *Trypanosoma brucei* editosome catalyzes the maturation of mitochondrial mRNAs through the insertion and deletion of uridylates and contains at least 16 stably associated proteins. We examined physical and functional associations among these proteins using three different approaches: purification of complexes via tagged editing ligases TbREL1 and TbREL2, comprehensive yeast two-hybrid analysis, and coimmunoprecipitation of recombinant proteins. A purified TbREL1 subcomplex catalyzed precleaved deletion editing in vitro, while a purified TbREL2 subcomplex catalyzed precleaved insertion editing in vitro. The TbREL1 subcomplex contained three to four proteins, including a putative exonuclease, and appeared to be coordinated by the zinc finger protein TbMP63. The TbREL2 subcomplex had a different composition, contained the TbMP57 terminal uridylyl transferase, and appeared to be coordinated by the TbMP81 zinc finger protein. This study provides insight into the molecular architecture of the editosome and supports the existence of separate subcomplexes for deletion and insertion editing.

Introduction

RNA editing in trypanosomatids remodels mitochondrial mRNAs through the insertion and deletion of uridylyl (U) residues, thereby creating functional transcripts (Stuart et al., 2002; Madison-Antenucci et al., 2002; Simpson et al., 2003). The sequence information for editing is provided by guide RNAs (gRNAs). At each site, the RNA editing process involves three sequential catalytic steps: (1) endonucleolytic cleavage, (2) U addition/removal, and (3) RNA ligation. Hence, four core activities are required for editing: endonuclease, U-specific exonuclease (ex-oUase), terminal U transferase (TUTase), and RNA ligase. It is likely that additional activities are involved, for example in RNA annealing and unwinding.

RNA editing is catalyzed by a multiprotein complex, the editosome. This complex has been purified by several labs (Pollard et al., 1992; Rusché et al., 1997; Madison-Antenucci and Hajduk, 2001; Panigrahi et al., 2001a, 2001b, 2003; Aphasizhev et al., 2003a). All purified complexes contained the core activities, sedimented around

20S on glycerol gradients, and performed at least one round of editing in vitro. However, the exact composition of the ~20S editosome is uncertain. We identified 16 proteins as stably associated components (Panigrahi et al., 2003) and a role in editing has been confirmed for five of these (TbREL1, TbMP63, TbMP81, TbMP44, and mHel61p) by gene knockout or knockdown experiments (Missel et al., 1997; Schnaufer et al., 2001; Huang et al., 2001, 2002; Drozdz et al., 2002; Wang et al., 2003). TbREL1 (*T. brucei* RNA editing ligase 1) is essential for editing and parasite viability and closely related to a second editing ligase, TbREL2 (McManus et al., 2001; Schnaufer et al., 2001; Huang et al., 2001), which does not appear to be essential (Drozdz et al., 2002). The related zinc finger proteins TbMP63 (*T. brucei* mitochondrial protein of 63 kDa) and TbMP81 (Panigrahi et al., 2001b) are both essential for editing and viability (Drozdz et al., 2002; Huang et al., 2002) and are also related to TbMP18, TbMP24, and TbMP42 (Panigrahi et al., 2001b, 2003). A second set of five related proteins (TbMP44, TbMP46, TbMP61, TbMP67, and TbMP90) has RNase III or RNA binding motifs (Panigrahi et al., 2003). TbMP99 and TbMP100 are also related and contain an exonuclease/endonuclease/phosphatase (exo/endo/phos) motif (Panigrahi et al., 2003). TbMP57 is a TUTase (Ernst et al., 2003). Other potential editosome proteins identified in our lab (TbMP33 and TbMP41) require further investigation (Panigrahi et al., unpublished data).

A second 108 kDa TbTUTase is related to TbMP57 but, although essential for editing (Aphasizhev et al., 2002), does not appear to be stably associated with the core editosome (Ernst et al., 2003; Aphasizhev et al., 2003a). A role in RNA editing has also been suggested for gBP21 (Blom et al., 2001; Müller et al., 2001), gBP25 (Blom et al., 2001), RBP16 (Pelletier et al., 2000), REAP-1 (Madison-Antenucci and Hajduk, 2001), and TBRGG1 (Vanhamme et al., 1998), all of which are able to bind gRNA and/or mRNA in vitro. We have not detected any of these proteins in our ~20S editosome preparations and their interaction with the core complex, if any, may be transient. A gBP21/gBP25 complex stimulates annealing of gRNA and mRNA in vitro and REAP-1 preferentially binds to preedited mRNA in vitro; thus, these proteins may be involved in delivering substrate RNA to the catalytic core complex (Madison-Antenucci and Hajduk, 2001; Müller et al., 2001; Aphasizhev et al., 2003b).

The purified ~20S complexes performed both insertion and deletion editing and blocks of sequence whose editing is specified by a single gRNA contain both insertion and deletion sites. Hence, insertion and deletion editing are generally thought to be catalyzed by the same complex. However, recent studies implied distinct functions for TbREL1 and TbREL2 in deletion and insertion editing, respectively, and raised the possibility that different sets of proteins may function in the two types of editing (Huang et al., 2001; Cruz-Reyes et al., 2002). The finding that other proteins of the editosome also occur as pairs or sets of proteins (Panigrahi et al., 2001b, 2003) reinforced this view.

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Through a combination of affinity purification of native complexes, yeast two-hybrid analysis, and coimmunoprecipitation experiments, we now have identified two distinct subcomplexes within the editosome that appear to consist of mutually exclusive sets of proteins. One subcomplex, containing TbREL1, TbMP63, TbMP99, and possibly TbMP18 catalyzed precleaved in vitro deletion editing while the other, containing TbREL2, TbMP81, and the TbMP57 TUTase catalyzed precleaved in vitro insertion editing. TbMP63 and TbMP81 are assigned central coordinating roles in their respective subcomplexes since they bind the catalytic proteins and are probably also involved in substrate recognition and binding. Finally, the present study suggests that TbMP99 is an exoUase.

Results

Tandem Affinity Purification of TbREL1 and TbREL2 Complexes

To compare protein complexes that contain TbREL1 versus TbREL2, we knocked in tetracycline (tc)-inducible versions of these ligase genes fused to C-terminal TAP tags (Rigaut et al., 1999). This ~21 kDa tag consists of a calmodulin binding peptide (CBP), a TEV protease cleavage site, and two protein A IgG binding peptides. Western analysis of the generated cell lines confirmed that expression of the tagged ligases was tightly regulated by tc and, when induced, did not affect cell growth (data not shown). Tagged editosomes were purified as outlined in Figure 1A. Tagged TbREL1 and TbREL2 were evident in Western blots of total lysates due to direct binding of the primary and secondary antibodies to the protein A peptides (Figure 1B, lanes 1 and 3). TbMP81, TbMP63, TbREL1, and TbMP42 editosome components for which we have monoclonal antibodies (mAbs) (Panigrahi et al., 2001b) were also detected (although less evident in lane 1). These four proteins were also readily detected in complexes that were bound by IgG Sepharose beads, washed, and eluted by digestion with TEV protease (Figure 1B, lanes 2 and 4). The cleaved-off protein A peptides remained bound to the column, reducing the size of the tagged proteins by ~16 kDa. This loss of the protein A peptides was evident in the reduced size of the tagged TbREL1 (TbREL1-CBP in Figure 1B, lane 2) and by the loss of reaction of tagged TbREL2 with the antibodies (Figure 1B, lane 4). The TbREL1 TEV eluate also contained TbREL1 with a size consistent with expression from the endogenous gene (Figure 1B, lane 2), suggesting the presence of tagged and endogenous TbREL1 in the eluted complexes. The amount of endogenous TbREL1 in the TEV eluate was variable between individual preparations and not always evident.

Separation of Two Different Tagged Ligase Complexes on Glycerol Gradients

TbREL1 and TbREL2 TEV eluates primarily sedimented as two broad peaks in 10%–30% glycerol gradients (Figures 1C and 1D). The ligases were detected by adenylation (upper panel) (Sabatini and Hajduk, 1995) and TbMP81, TbMP63, TbREL1, and TbMP42 by Western blotting with mAbs (lower panels). One peak sedimented at ~20S for both TbREL1 and TbREL2, cosedimented with TbMP81, TbMP63, and TbMP42 (fractions 13–19), and corresponded to the sedimentation of editosomes capable of in vitro editing (Pollard et al., 1992; Corell et al., 1996; Rusché et al., 1997). A second ligase peak sedimented at 5-10S (fractions 3–9). Most tagged and untagged TbREL1 was in the 5-10S peak (e.g., Figure 1C), although this varied somewhat between experiments. Some untagged REL1 was also often present in the ~20S peak, as recently described for similarly tandem affinity-purified complexes from *Leishmania tarentolae* (Aphasizhev et al., 2003a), raising the possibility of more than one TbREL1 molecule in at least a fraction of the ~20S editosomes. The distribution of TbMP63 overlapped the 5-10S TbREL1 peak, suggesting that this protein may be associated with some TbREL1 in subcomplexes. Importantly, in contrast to the ~20S complexes, the 5-10S complexes from cells expressing TbREL1-TAP did not contain detectable amounts of TbREL2 and, similarly, the 5-10S complexes from TbREL2-TAP cells did not contain detectable TbREL1 (Figure 1D). Adenylated TbREL2-CBP comigrated with endogenous TbREL1 due to the retained CBP portion of the tag (Figure 1D, upper panel). However, analysis with α-TbREL1 mAb clearly showed the absence of TbREL1 from the TbREL2 5-10S complexes (Figure 1D, lower panels). Substantial TbMP81 cofractionated in this region of the gradient. The identity of the smaller adenylatable polypeptide in the 5-10S region of TbREL2-TAP gradients is unknown. It is too large to be endogenous TbREL2 but may be a proteolytic product of the tagged TbREL2. We did not determine if the 5-10S complexes were present in vivo in parasites expressing TAP-tagged ligases or whether they resulted from the purification procedure. Ligase complexes of comparable size have been reported in some preparations from *T. brucei* and *L. tarentolae* (Corell et al., 1996; Peris et al., 1997) and in purified *L. tarentolae* REL1 complexes (Aphasizhev et al., 2003a) but were absent from other preparations (Sabatini and Hajduk, 1995; Rusché et al., 1997).

Compositions of TbREL1 and TbREL2 5-10S Complexes

Analysis of pooled 5-10S and ~20S fractions from each glycerol gradient after additional purification over calmodulin resin revealed distinct and largely mutually exclusive compositions of subcomplexes derived from TbREL1-TAP versus TbREL2-TAP cells (Figure 1E). Western analyses using seven available mAbs and polyclonal antisera revealed all target proteins in ~20S fractions from TbREL1-TAP and TbREL2-TAP cells. Mass spectrometric analyses revealed that the composition of the TbREL1-TAP ~20S complex is similar to previously identified editosomes, as reported elsewhere (Panigrahi et al., 2003), and also is similar to the recently tandem affinity-purified editosome from *L. tarentolae* (Aphasizhev et al., 2003a). However, there were clear differences between the two 5-10S fractions. The TbREL1 5-10S fraction contained TbMP63 and small amounts of TbMP18 (Figure 1E). The large amount of untagged TbREL1 present in the 5-10S region of the glycerol gradient (Figure 1C) was not recovered in this further purified 5-10S material, indicating, if any, an unstable association with tagged TbREL1. Silver-stained SDS-PAGE gels revealed
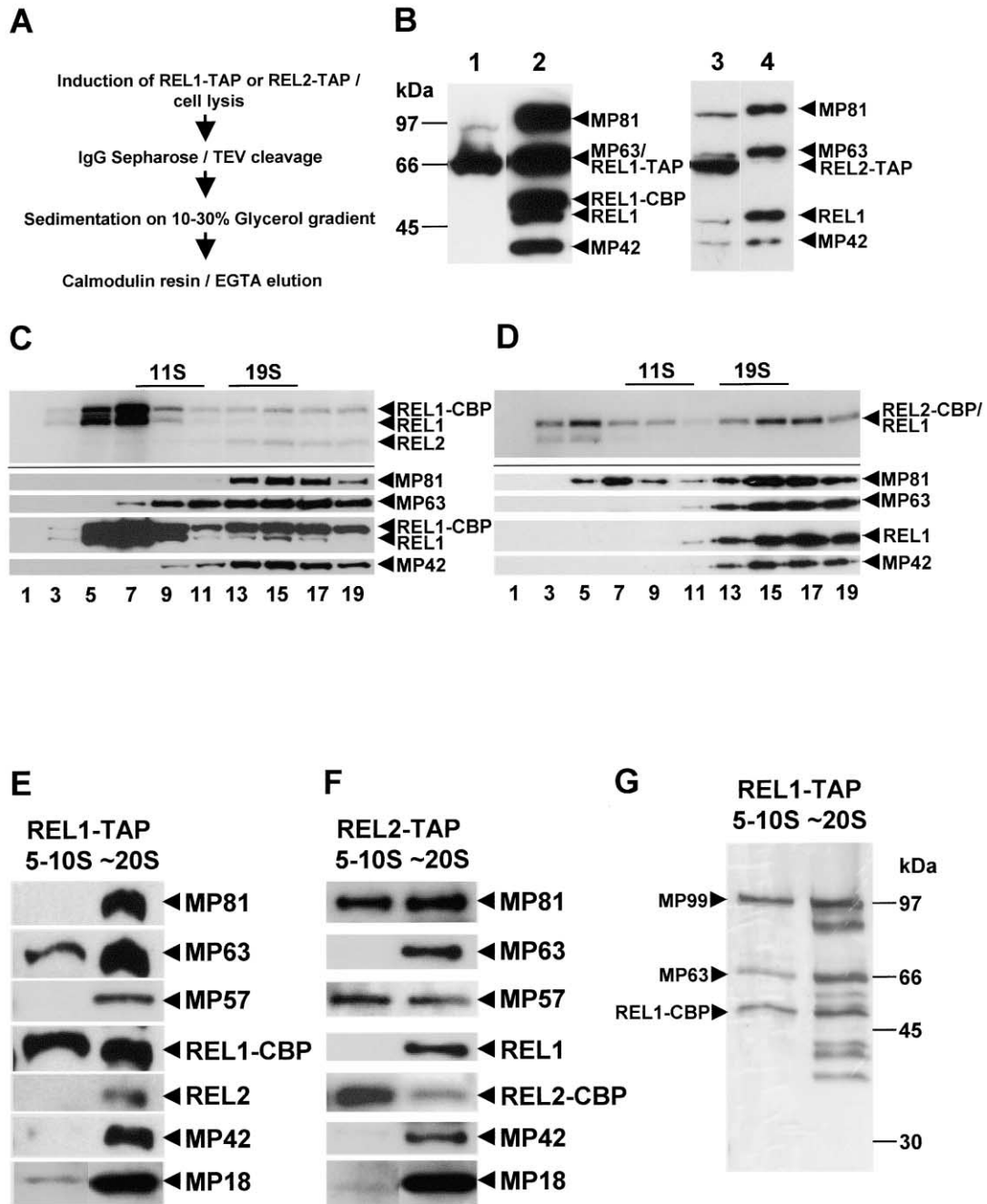
Figure 1. Purification and Analysis of Ligase-Associated Complexes

(A) Purification strategy.

(B) First purification step. Left panel, TbREL1-TAP; right panel, TbREL2-TAP. Total lysates (lanes 1 and 3) and TEV eluates (lanes 2 and 4) were fractionated by SDS-PAGE, blotted, and probed with mAbs against TbMP81, TbMP63, TbREL1, and TbMP42.

(C) Fractionation of TbREL1-TAP TEV eluates from (B), lane 2, on 10%–30% glycerol gradients. Fractions were collected from the top of the gradients. Aliquots from odd-numbered fractions were analyzed by adenylation with α-^{32}P-ATP and SDS-PAGE (upper panel), and by SDS-PAGE followed by immunoblot analysis with mAbs as above (lower panels).

(D) Same analysis as described in (C), starting with TbREL2-TAP TEV eluates from (B), lane 4.

(E and F) Pooled fractions representing 5-10S and ~20S TbREL1 (E) and TbREL2 (F) complexes from glycerol gradients such as shown in (C) and (D) were further purified over calmodulin resin, separated by SDS-PAGE, and analyzed with the mAbs and polyclonal antisera indicated.

(F) Analysis of tandem affinity-purified 5-10S and ~20S TbREL1 complexes by SDS-PAGE and silver-staining. The three bands visible in the 5-10S complex were identified by tandem mass spectrometry. The data shown are representative examples obtained from multiple purifications. Experiments were reproducible but showed variability in yield and ratio of 5-10S versus ~20S complexes.

an additional major protein of ∼100 kDa (Figure 1G) and mass spectrometric analysis of the excised band identified this protein as TbMP99 and the two other bands as TbMP63 and TbREL1, consistent with the immunoanalyses. Western analysis of the TbREL2 5-10S fraction revealed TbMP81 and TbMP57 and small amounts of TbMP18 and TbMP42 (Figure 1F). SDS-PAGE/silver-staining analysis of the TbREL2 5-10S fraction was not possible due to poor binding of TbREL2-CBP to the calmodulin resin and hence low yield. These results indicate the presence of two stable and separable editosome subcomplexes with distinct compositions. Although we cannot exclude the presence of other proteins in the two subcomplexes due the lack of antibodies and, in the case of TbREL2, sufficient protein, one subcomplex appeared to primarily contain TbREL1, TbMP63, and TbMP99, while the second subcomplex primarily contained TbREL2, TbMP81, and TbMP57. The simplest possibility is that there is a single subcomplex for each ligase. However, these subcomplexes could be heterogeneous with respect to protein composition since the profiles of TbREL1 and TbMP63 did not directly overlap in the 5-10S region, although this could reflect TbREL1 aggregates or association with other proteins and perhaps less efficient binding by the calmodulin column. Both complexes appeared to contain small amounts of TbMP18 and perhaps TbMP42.

In Vitro Editing by TbREL1 and TbREL2 Complexes
The ∼20S complexes catalyzed a full round of editing in vitro and thus contain all four core editing activities, i.e., endonuclease, exoUase/TUTase, and ligase as reported elsewhere (Panigrahi et al., 2003). However, we did not detect full round editing or endonuclease activity in 5-10S TbREL1 or TbREL2 complexes (data not shown), which may indicate the absence of endonuclease activity or reflect limited sensitivity. Next, we assayed precleaved in vitro insertion and deletion editing. These assays provide insertion or deletion mRNA substrates as separate synthetic 5′ and 3′ fragments and are therefore independent from endonuclease activity (Igo et al., 2000, 2002). The 5-10S TbREL1 complexes were active in precleaved deletion editing while the 5-10S TbREL2 complexes were active in precleaved insertion editing (Figure 2).

Two adenosines in the gRNA of the precleaved insertion substrate specified the addition of two U's to the labeled 5′ fragment. The ∼20S TbREL1 and TbREL2 complexes both produced accurately edited RNA with two inserted U's although the TbREL2 activity was lower (Figure 2A, lanes 4 and 6). The +2U product was evident for the TbREL1 complex, but not for the less active TbREL2 complex, and both complexes also ligated the input mRNA fragments. The minor band above the input and the input ligation products was due to a small amount of 5′ mRNA fragment with an additional nucleotide, a byproduct of in vitro synthesis (Figure 2A, lane 1). In contrast, the TbREL2 5-10S complex but not the TbREL1 5-10S complex catalyzed precleaved insertion editing (Figure 2A, lanes 3 and 5). The +2U product and edited RNA were prominent but ligated input was not evident with the TbREL2 5-10S complex. Neither U addition nor edited products were detected with the TbREL1

5-10S complex; however, ligated input was detected. The precleaved deletion substrate specified the removal of four unpaired U's from the labeled 5′ mRNA fragment (Figure 2B, upper panel). The ∼20S TbREL1 and TbREL2 complexes had comparable activity in this assay. Both removed up to four U's and ligated the -4U product to produce edited RNA and also ligated the input mRNA fragments. However, the TbREL1 5-10S complexes produced accurately edited RNA while the 5-10S TbREL2 complexes catalyzed neither U removal nor RNA ligation. The minor band migrating just above the input in Figure 2B, lanes 2–4, is a common side product of this assay (Igo et al., 2002) that may represent circularized substrate and is not the result of U addition since UTP was absent from the deletion reactions.

These results demonstrated that the 5-10S TbREL1 complex contained specific 3′ exoUase and ligase activities required for deletion editing, whereas the 5-10S TbREL2 complex contained specific TUTase and ligase activities required for insertion editing. The primary presence of TbREL1, TbMP63, TbMP99, and, to a lesser extent, TbMP18 in the 5-10S TbREL1 complex suggests that these three proteins are sufficient to perform precleaved deletion editing. It follows that one of these proteins possesses exoUase activity, not previously assigned to any protein. The presence of an exo/endo/phos motif in TbMP99 (Panigrahi et al., 2003) identifies this protein as the most likely candidate. Similarly, the primary presence of TbREL2, TbMP81, and TbMP57 in the 5-10S TbREL2 complexes suggests that these three proteins are sufficient to perform precleaved insertion editing. As will be reported elsewhere (Ernst et al., 2003), recombinant TbMP57 has 3′ TUTase activity. Interestingly, the 5-10S TbREL1 complex catalyzed the ligation of input mRNA fragments while the 5-10S TbREL2 complex did not, although it actively ligated the two U addition products. Hence, TbREL1 may have less stringent substrate requirements than TbREL2.

Yeast Two-Hybrid Analysis
In order to identify binary protein-protein interactions within the editosome, we performed a comprehensive yeast two-hybrid screen (Fields and Song, 1994). The coding regions of editosome components and candidates were fused to Gal4 activation (AD) and DNA binding domains (BD) of pOAD and pOBD2 plasmids, respectively. Yeast *PCL2* and *PCL9* genes were included as positive controls in the AD set and *PHO85* in the BD set since the protein pairs PCL2/PHO85 and PCL9/PHO85 interact (Uetz et al., 2000). Plasmids without insert served as negative controls. The fusion plasmids were transformed into haploid yeast strains and the resulting transformants were mated in all possible combinations. Interactions were identified by growth of diploids on plates lacking histidine (Figure 3A). BD fusions of TbMP46, TbMP63, TbMP81, and gBP25 were self-activating as indicated by growth in all pair-wise combinations (Figure 3A and see below). Hence, plates were supplemented with up to 30 mM 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of the HIS3 auxotrophic marker, to increase the stringency of the screen. Interactions were scored as positive when growth over background occurred in at least two out of three independent screens.
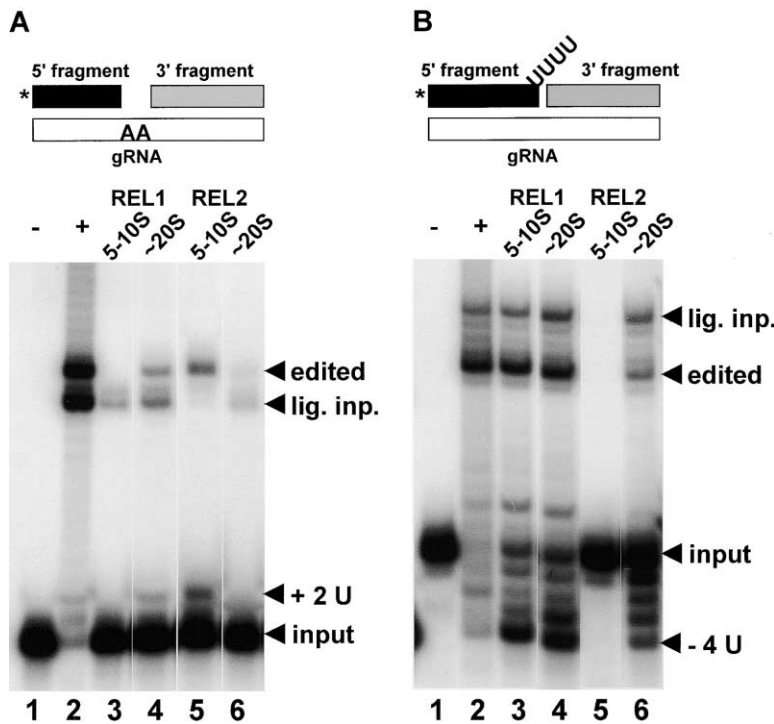
Figure 2. Analysis of TbREL1-TAP and TbREL2-TAP 5-10S and ~20S Fractions by In Vitro Editing Assays

(A) Precleaved insertion editing assay. -, no extract (negative control); +, purified mitochondrial extract (Panigrahi et al., 2001a) (positive control). Editing intermediates and end products are indicated. Asterisk, radioactive 5′ label of the 5′ mRNA fragment.
(B) Precleaved deletion editing assay. Lanes and labels are as in (A).

The screen identified six interactions linking seven proteins, indicated by boxes with solid lines in Figure 3A and summarized in Figure 3B. All interactions involved either TbMP63 or TbMP81. TbMP63 interacted with both TbREL1 and TbREL2, with TbMP99, and, variably between experiments, with TbMP18 (dotted box, Figure 3A). Interactions with TbMP63 could only be scored when that gene was in the AD configuration, since its BD fusion was self-activating up to at least 30 mM 3-AT, the highest concentration tested (Figure 3A). TbMP81 interacted with TbMP57 and TbMP18 in both configurations. Although the BD fusion of TbMP81 was self-activating, inclusion of 2.5 mM 3-AT allowed the clear identification of these interactions (Figure 3A). The AD fusion of TbMP81 showed clear interaction with TbREL2. Although we observed differences in growth among the positive interactions, these did not allow direct conclusions on the strength of the particular interaction since the assay also depends on the efficiency of protein expression, proper folding, and the effect of the domain fusion on protein structure.

Coimmunoprecipitation of Recombinant Proteins
The binding specificity of TbMP63 and TbMP81 to the two editing ligases was further tested by in vitro immunoprecipitation experiments using recombinant, ^{35}S-methionine-labeled proteins and specific mAbs (Figure 4A). As previously reported (Panigrahi et al., 2001b), a mAb specific for TbMP63 coimmunoprecipitated TbREL1 (lane 1), consistent with the results of the two-hybrid analysis. However, contrary to the two-hybrid data, TbREL2 did not coimmunoprecipitate with TbMP63 (lane 2). A mAb specific for TbMP81 coimmunoprecipitated TbREL2 (lane 7), again in accordance with the two-hybrid analysis. Small amounts of TbREL1 were also

coimmunoprecipitated, but those were not above background levels obtained in the absence of TbMP81 (compare lanes 6 and 9).

Separate immunoprecipitates of TbREL2-HIS, TbMP81-HIS, and TbMP63, individually as well as in combination, were assayed for ligase activity using a double stranded (ds) RNA substrate (Igo et al., 2000) (Figure 4B). The presence of TbMP81 stimulated the activity of TbREL2 ~11-fold. TbMP63, which did not coimmunoprecipitate with TbREL2 (Figure 4A) but interacted with it in the two-hybrid analysis (Figure 3), stimulated its activity only ~2-fold. A duplicate experiment gave similar results. These data indicate that the pairs TbREL1/TbMP63 and TbREL2/TbMP81 interact physically and indicate that these interactions, at least in the case of TbREL2/TbMP81, can enhance activity. We did not find a similar stimulation for TbREL1/TbMP63, which might be due to interference of the antibodies with any functional interaction or a lack thereof.

Predicted Secondary Structures of RNA Editing Ligases, TbMP63, and TbMP81
We compared secondary structure predictions for the editing ligases and their binding partners TbMP63 and TbMP81 with the known secondary structures of two DNA ligases (whose crystal structures have been determined) and *E. coli* single strand binding (SSB) protein (Figure 5). Only the C-terminal portions of TbMP63 and TbMP81 are shown for convenience and are aligned with TbMP42 and TbMP18, which have C-terminal sequence similarity to TbMP63 and TbMP81 (Panigrahi et al., 2001b). Typically, DNA ligases and RNA capping enzymes feature a modular domain architecture with at least adenylation and OB fold domains (Doherty and Suh, 2000). The adenylation domain contains conserved
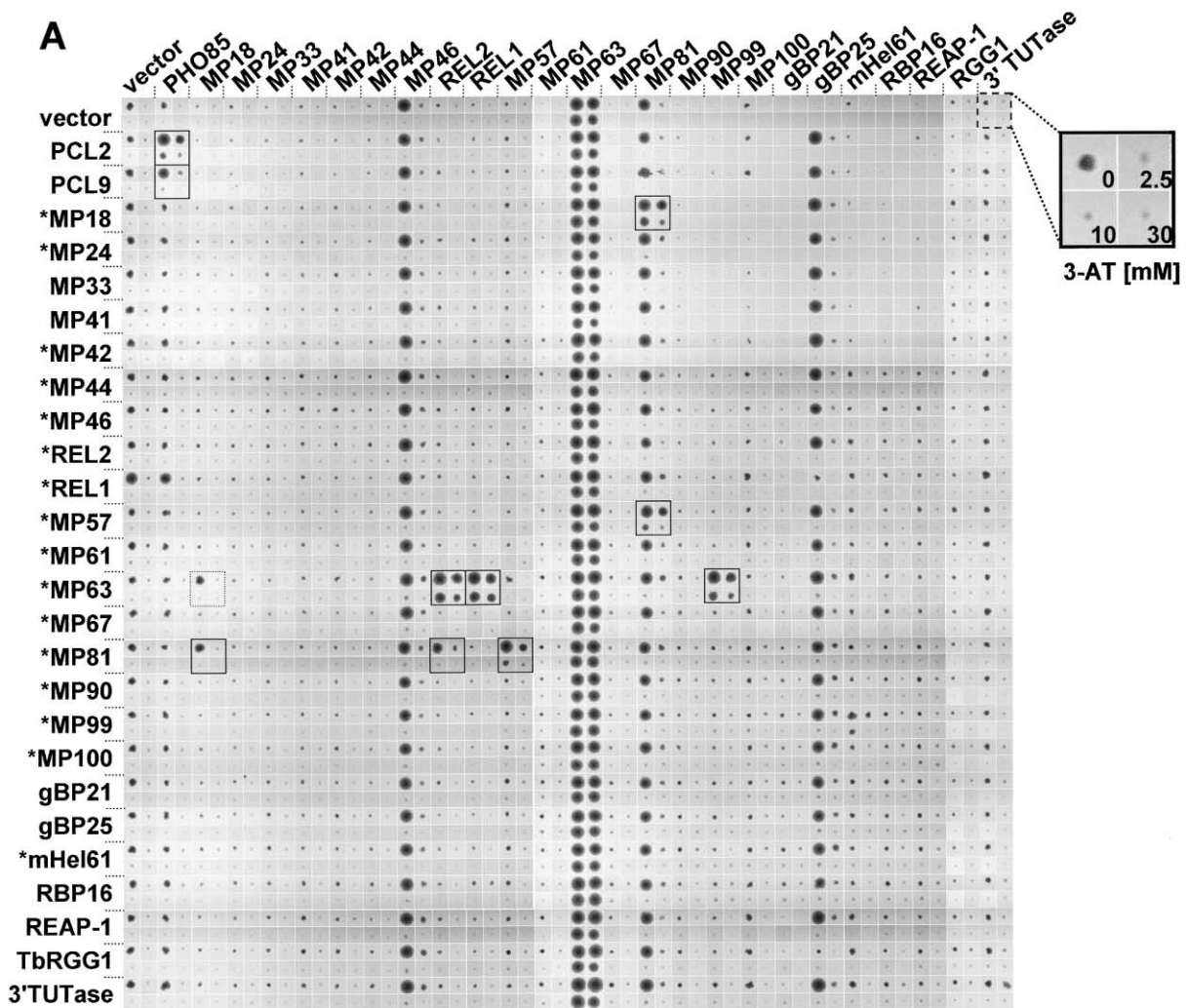
A

B

AD	BD
TbMP18	TbMP81
TbMP57	TbMP81
TbMP63	TbREL2, TbREL1, TbMP99
TbMP81	TbMP18, TbREL2, TbMP57

Figure 3. Yeast Two-Hybrid Analysis of Protein-Protein Interactions

(A) Array of 27 × 26 protein pairs assayed in the screen. Diploid yeast strains representing all pair-wise combinations were plated on medium lacking Trp, Leu, and His. The figure represents a composite of plates supplemented with 0, 2.5, 10, or 30 mM 3-AT. AD fusions in rows, BD fusions in columns. Asterisks indicate stably associated components of the RNA editing complex (see text). Yeast proteins PCL2/PHO85 and PCL9/PHO85 represent positive controls.

(B) Summary of interactions identified in (A).

sequence motifs I–IV while motif V links the adenylation domain to the OB fold domain, which contains motif VI and has a five-stranded β sheet structure, coiled to form a closed β barrel and capped by an α helix, usually located between the third and fourth strand (Suck, 1997). OB fold domains are found in a wide variety of protein families that bind to single stranded (ss) and/or ds nucleic acids (Suck, 1997).

The comparisons predicted structural similarity of TbREL1 and TbREL2 with the adenylation domain of ChV and T7 DNA ligases in the region up to and including motif V (Figure 5). The C-terminal regions of TbREL1 and TbREL2 have predicted structures that consist predominantly of α helices, in contrast to the OB fold domain of the ChV and T7 ligases. Intriguingly, the predicted structures of the C-terminal regions of TbMP81,
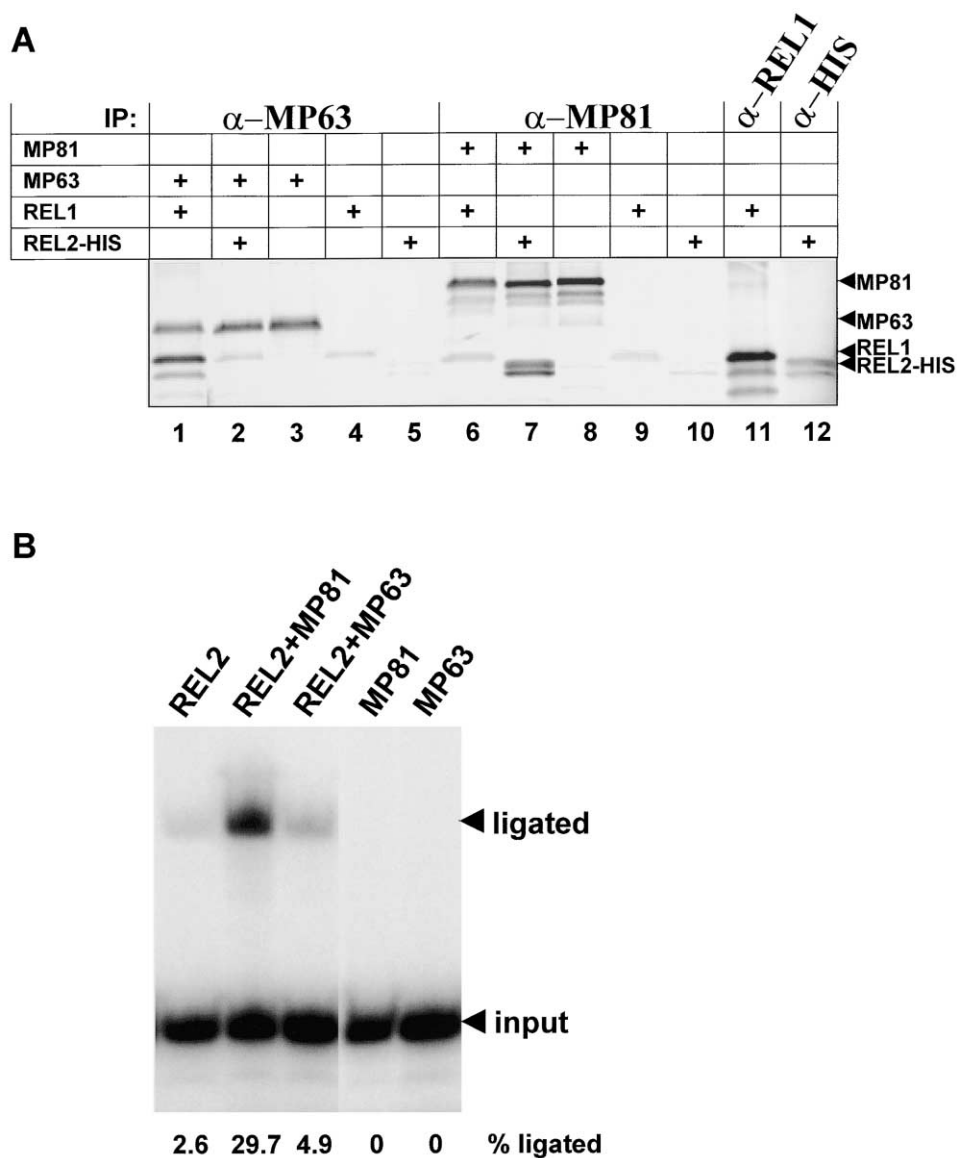
A

IP:	α–MP63					α–MP81					α–REL1	α–HIS
MP81						+	+	+				
MP63	+	+	+									
REL1	+			+		+			+		+	
REL2-HIS		+			+		+			+		+

◀ MP81
◀ MP63
◀ REL1
◀ REL2-HIS

1 2 3 4 5 6 7 8 9 10 11 12

B

◀ ligated

◀ input

2.6 29.7 4.9 0 0 % ligated

Figure 4. Physical and Functional Interactions between Editing Ligases and TbMP63/TbMP81 Zinc Finger Proteins

(A) Coimmunoprecipitation experiments. Proteins were in vitro transcribed/translated in the presence of ^{35}S-methionine, mixed where indicated, and precipitated with the mAbs indicated.

(B) In vitro ligation assay. In vitro transcribed/translated proteins were immunoprecipitated with α-6xHis (TbREL2, TbMP81) or α-TbMP63 mAbs and, individually or in combination, assayed for ligation of a nicked ds RNA substrate. Percent ligation efficiencies of the individual reactions are indicated.

TbMP63, TbMP42, and TbMP18 have a high degree of similarity to OB fold domains. Computer searches against motif databases supported these predictions. ProfileScan (http://hits.isb-sib.ch/cgi-bin/PFSCAN) indicated similarity of the C-terminal region of TbMP81 and TbMP63 to Pfam motifs SSB and TRNA_ANTI, respectively, and the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) indicated similarity of the corresponding region of TbMP18 to the SSB domain, all of which represent OB folds.

Discussion

This study provides insight into the architecture of the *T. brucei* editosome and provides strong evidence for

a physical and functional separation of deletion and insertion RNA editing activities in subcomplexes, as summarized in Figure 6. Three different approaches, yeast two-hybrid analysis, coimmunoprecipitation studies, and analyses of purified complexes using affinity-tagged versions of the two RNA editing ligases are consistent with each other and are also consistent with and expand on published genetic and biochemical studies (see below). Our data provide direct evidence that the TbREL1 ligase along with TbMP63, TbMP99, and possibly TbMP18 forms a subcomplex that specifically catalyzes precleaved U-deletion editing. These results suggest that one of these proteins, most likely TbMP99, which has an exo/endo/phos motif, is an exoUase. The proteins responsible for the ligase and exoUase activi-
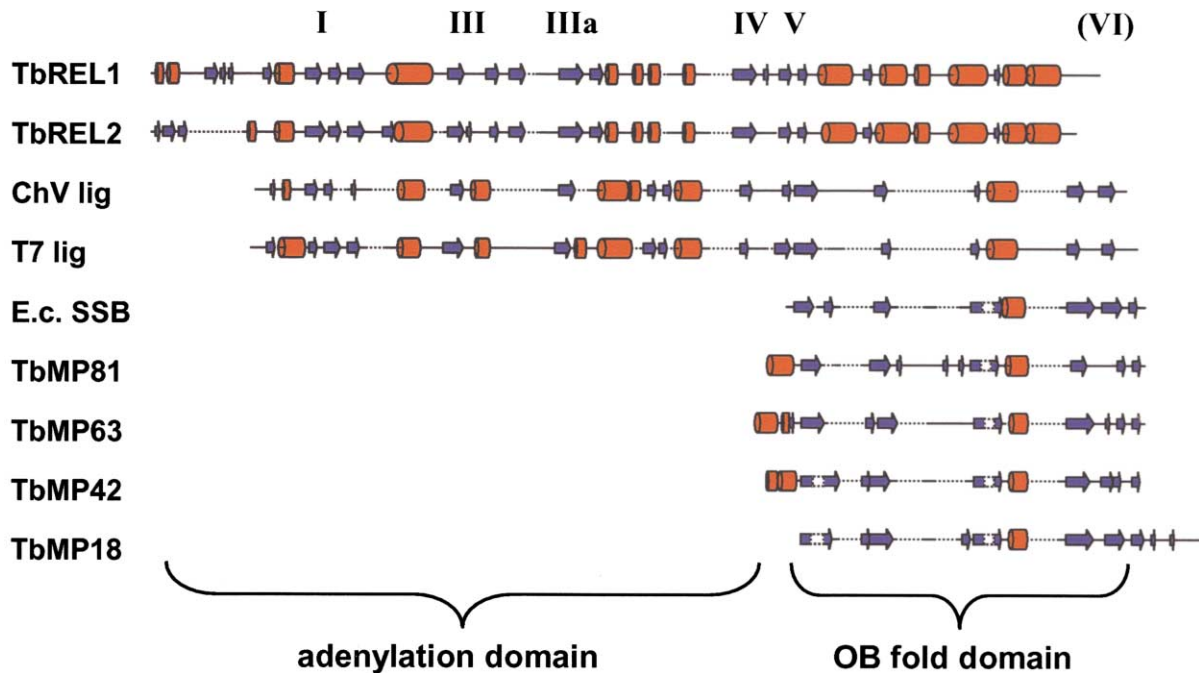
Figure 5. Secondary Structure Analysis of RNA Editing Ligases and Four Related Editosome Components

Secondary structures were predicted with PROFsec and aligned with the structures for *E. coli* SSB and DNA ligases ChV and T7, which were taken from PDB files 1EYG, 1FVI and 1A0I, respectively. Numbers I–V correspond to the nucleotidyl transferase motifs shared between the RNA and DNA ligases. Motif VI has been identified in the DNA ligases only. α helices are represented by red cylinders, β strands are represented by blue arrows. Dotted lines indicate gaps in the alignment.

ties appear to be bound within this deletion subcomplex by the TbMP63 zinc finger protein. The predicted presence of an OB fold domain in TbMP63 suggests that it may have roles in the recognition and/or binding of RNA substrates for deletion editing. Similarly, TbREL2 ligase along with TbMP81 and TbMP57 forms a subcomplex that specifically catalyzes precleaved U-insertion editing. The recent finding that recombinant TbMP57 is a TUTase (Ernst et al., 2003) is consistent with these data. Similar to TbMP63, the zinc finger protein TbMP81 binds both TbREL2 and TbMP57 proteins and has a predicted OB fold domain and thus may coordinate the ligase and U-addition activities for insertion editing. While a trimeric structure of the subcomplexes as shown in Figure 6 represents the simplest model consistent with our results and other published data, we cannot exclude the existence of alternative or more complex associations. For instance, some proteins (e.g., TbMP18, TbMP42, or other, unidentified proteins) may be more loosely associated with either subcomplex and therefore be lost in the majority of the purified subcomplexes or the subcomplexes may even have alternative compositions. No endonuclease activity was found associated with either subcomplex suggesting that this function is provided by one or more other proteins. We propose a model below for a novel interaction between a ligase and its binding partner that enhances the specificity of editing and coordinates the catalytic steps of this process.

The data presented here are consistent with results obtained in recent genetic studies. Ablation of TbMP63 by RNAi resulted in disruption of complexes and loss of TbREL1 (Huang et al., 2002). Although both TUTase

and exoUase activities were still present in TbMP63-depleted cells, the characteristics of U-deletion were altered, raising the possibility of multiple exoUase activities. Ablation of TbMP81 expression by RNA interference (RNAi) resulted in loss of TbREL2 from the editing complex and the purified complexes were preferentially impaired in U-insertion-related in vitro editing activities (Drozdz et al., 2002). Despite the size change that might be expected from a loss of TbMP81, possibly along with TbREL2 and TbMP57, these editosomes still sedimented around 20S. However, the glycerol gradients in those experiments had relatively low resolution and more study is needed to further assess the structural consequences of loss of TbMP81. Expression of TAP-tagged REL1 complexes in *L. tarentolae* also resulted in 5-10S REL1 subcomplexes that contained LC-4 (the ortholog of TbMP63) and LC-3, a protein that was not further characterized (Aphasizhev et al., 2003a). Interestingly, LC-3 has substantial homology to TbMP99 but lacks the C-terminal exo/endo/phos motif (R.S., unpublished data). It had not been reported whether the LtREL1 5-10S complex had exonuclease activity.

Our data provide direct evidence for distinct functions of TbREL1 and TbREL2 in deletion and insertion editing, respectively. This distinction had been suggested on biochemical evidence showing enzymatic differences in the cleavage and ligation steps between deletion and insertion editing: in vitro cleavage steps were inversely affected by ADP or ATP concentrations (Cruz-Reyes et al., 1998) and the two RNA editing ligases became adenylated at different ATP concentrations, which was paralleled by differential responses of in vitro deletion
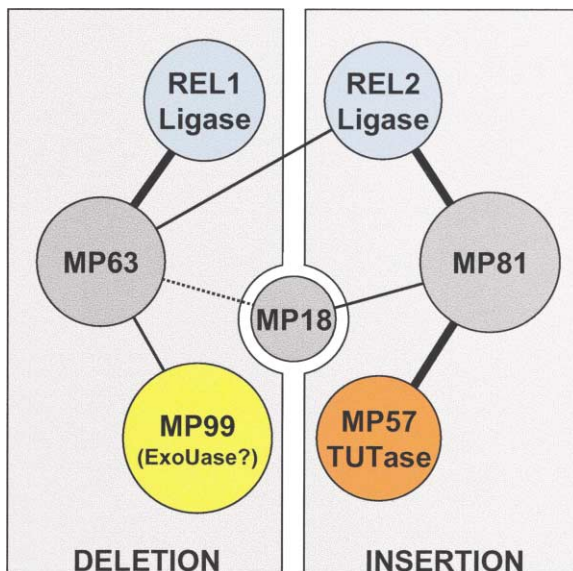
Figure 6. Summary of Interaction Data and Integration with Published Data

Related proteins are indicated by identical color. Thick solid lines indicate interactions shown by yeast two-hybrid, coimmunoprecipitation, and genetic studies. Thin solid lines indicate reproducible interactions in the yeast two-hybrid assay. Dotted lines indicate interactions that showed some variability in the yeast-two-hybrid assay. See text for references.

and insertion editing to ATP (Cruz-Reyes et al., 2002). However, this functional distinction is probably not absolute, since ablation of TbREL2 by RNAi had no effect on either in vivo or in vitro RNA editing, indicating that TbREL1, at least in the absence of TbREL2, can also function in insertion editing (Drozdz et al., 2002). In contrast, ablation of TbREL1 resulted in loss of editing and was lethal, suggesting that TbREL2 cannot function in deletion editing (Schnaufer et al., 2001; Huang et al., 2001).

Compensation for the loss of TbREL2 by TbREL1 might occur by physical replacement of TbREL2 in the insertion subcomplex or by TbREL1 providing ligation activity for insertional editing from its normal position within the deletion subcomplex. The first possibility implies that TbREL1 can interact with TbMP81. We did not find evidence for such an interaction but instead found that TbREL2 interacted with TbMP63 in the two-hybrid analysis (Figure 3). However, our separation of the two ligase complexes allowed us to reveal differences in their catalytic characteristics: TbREL1 5-10S complexes ligated RNAs with a gap or overhang (Figures 2A and 2B, lanes 3) while TbREL2 5-10S complexes required a perfect duplex, i.e., a nicked ds RNA (Figures 2A and 2B, lanes 5). These differences may reflect the differences in affinity for ATP between the two ligases (Cruz-Reyes et al., 2002) and in catalytic specificity between extracts from cells with downregulated expression of TbREL1 or TbREL2 (Huang et al., 2001) (S.S.P. et al., unpublished data). Thus, as previously suggested (Cruz-Reyes et al., 2002), the apparently relaxed specificity of TbREL1 compared to TbREL2 might enable the former to compensate loss of the latter, but not vice versa.

Our data suggest that TbMP63 and TbMP81 not only bind the catalysts but may also have roles in substrate recognition and binding and thus specificity. TbMP81 stimulates activity of TbMP57 (Ernst et al., 2003) and data presented here suggest that it also stimulates activity of TbREL2. The structural predictions suggested that TbMP63 and TbMP81 C-terminal regions assume an OB fold-like structure (Figure 5). An OB fold domain is present in all DNA ligases and RNA capping enzymes (Doherty and Suh, 2000) and a positively charged cleft that is formed by the conjunction of the catalytic (adenylation) and OB fold domains has been proposed to function in substrate binding in DNA ligases (Figure 7A) (Subramanya et al., 1996). The RNA editing ligases contain the catalytic domain but appear to lack the C-terminal OB fold domain (Figure 5). We propose that C-terminal regions of TbMP63 and TbMP81 provide the OB fold domain in *trans* to TbREL1 and TbREL2, respectively, as illustrated in Figure 7B. It is attractive to assume that, as shown in the model, the C-terminal domains of the editing ligases interact with their binding partners.

Comparison to other ligases suggests how this arrangement might coordinate editing ligase function. Ligation occurs by (1) adenylation of the ligase, (2) transfer of the AMP to the 5′ terminus of the DNA or RNA, and (3) joining of the two polynucleotides with release of AMP. While T7 DNA ligase with its OB fold domain deleted retained adenylation and ligation activities (Doherty and Wigley, 1999), both were stimulated by addition of the OB fold domain in *trans*. The adenylation and OB fold domains of T7 DNA ligase have also been suggested to function together for substrate recognition and strand joining (Doherty and Suh, 2000; Odell et al., 2000). The OB fold domain was proposed to rotate upon adenylation and expose the DNA binding cleft formed by the two domains, thus providing a conformational switch (Figure 7A). Moreover, specific recognition of nicked DNA was suggested to entail interaction between the two domains (Doherty and Wigley, 1999). These features of the T7 DNA ligase suggest that TbMP63 and TbMP81 might control the activity of their ligase binding partners through their OB fold domains (Figure 7B). Some DNA ligases, e.g., human DNA ligase III, contain zinc fingers that contribute to the specific binding of nicked DNA (Mackey et al., 1999), suggesting that the zinc fingers of TbMP63 and TbMP81 might have a similar function.

TbMP63 and TbMP81 not only bind the ligases but also bind the TUTase (TbMP57) and exoUase (potentially TbMP99) enzymes (Figures 6 and 7C). This arrangement could function to coordinate steps of editing. Conformational switches of the TbMP81 and TbMP63 OB fold domains during insertional and deletional editing, respectively, would ensure sequential enzymatic steps, illustrated in a conceptual "toggle" model in Figure 7D. In this model, the conformational changes of TbMP63 and TbMP81 are represented as toggles, which control the access of substrate to the TUTase, exoUase, and two RNA ligase catalytic domains. For example, a substrate with a deletion and insertion site would first encounter the catalytic complex with the toggles in the neutral position and editing would be initiated by endonucleolytic cleavage of the deletion site by an unidenti-
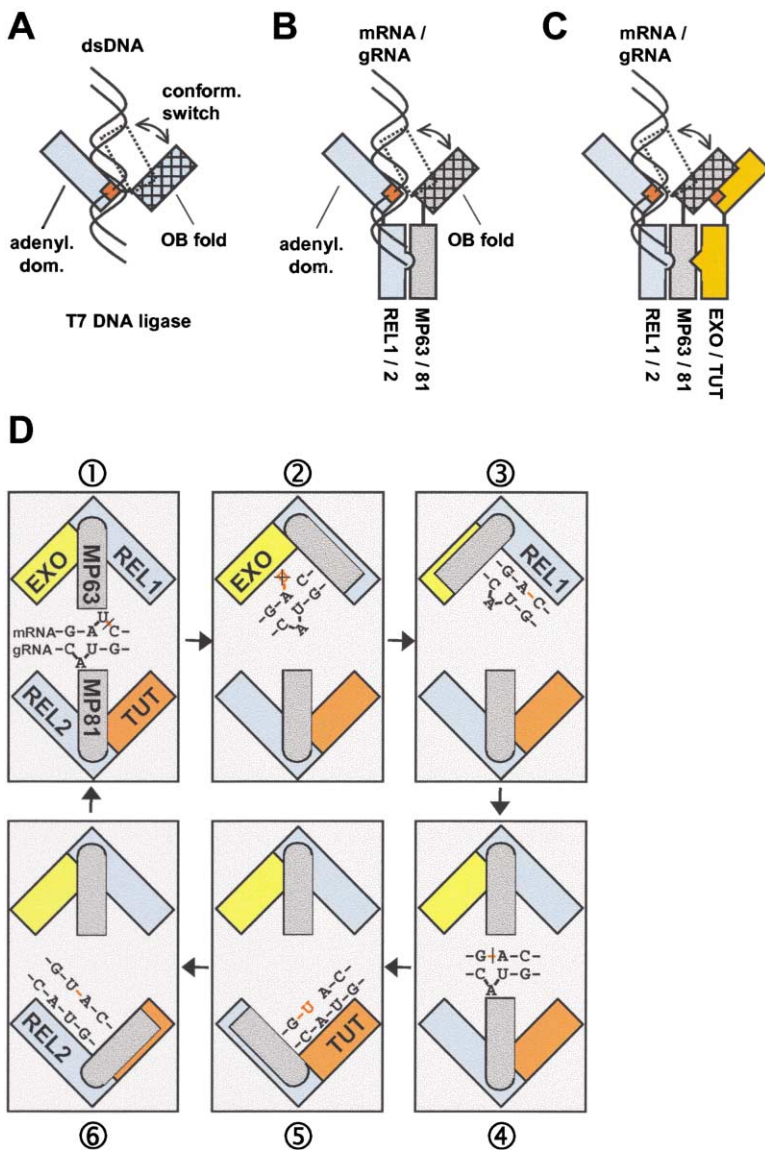
Figure 7. Domain Architecture Model

(A) Domain architecture of T7 DNA ligase. The OB fold domain is crosshatched and its conformational switch that allows substrate binding is indicated (see text). The nicked ds DNA substrate was positioned according to Doherty and Suh (2000).

(B) Proposed domain architecture for the deletion and insertion subcomplexes of the RNA editing complex. Blue, ligase (REL); gray, TbMP63/TbMP81; orange, exoUase/TUTase (EXO/TUT). Active sites are indicated in red. The two-domain structures of the proteins are hypothetical.

(C) "Toggle" model for the control of sequential enzymatic steps during editing. Enzymatic changes of the mRNA substrate are indicated in red. See text for details.

fied enzyme (step 1). Subsequent conformational changes of the TbMP63 and TbMP81 OB fold domains (steps 2–6) would then sequentially form the exoUase, TbREL1, TUTase, and TbREL2 substrate binding and catalytic sites, which would edit the substrate as specified by the gRNA. In reality, the conformational switches may represent stabilization of dynamic conformations as a result of binding site recognition of its specific substrate.

Separation of adenylation and OB fold domains into two different proteins would be unprecedented among ligases but it may not be restricted to the RNA editing ligases since the predicted secondary structure of bacteriophage RNA ligase T4Rnl2 (Ho and Shuman, 2002) also suggests the absence of a C-terminal OB fold domain (data not shown).

The ability to isolate the 5-10S TbREL1 and TbREL2 subcomplexes indicates that the intermolecular interactions within each subcomplex are probably more stable than the interactions that hold the subcomplexes in the

editosome. The latter interactions might involve other proteins such as TbMP44, the loss of which results in disruption of editosomes (Wang et al., 2003). They may also involve TbMP63, which may be able to interact with both ligases simultaneously (Figure 6), or TbMP18, which showed some association with both 5-10S subcomplexes (Figures 1E and 6).

Of the 16 confirmed editosome proteins (Panigrahi et al., 2003), our two-hybrid data have linked 7. Some protein-protein interactions within the editosome likely remained undetected since some fusion proteins might not be expressed at sufficient levels or properly folded and some interactions might require additional molecules, such as other proteins or RNAs. Our editosome interaction map accounts for proteins that catalyze U addition, U removal, and ligase activities for insertion and deletion editing but not the endonuclease activity, although we have identified several potential endonucleases in purified editosomes (Panigrahi et al., 2003). The presence of more than one endonuclease

would not be surprising given the finding of pairs and sets of related editosome proteins (Schnaufer et al., 2001; Panigrahi et al., 2001b, 2003) and the biochemical differences between the cleavage steps of deletion and insertion editing (Cruz-Reyes et al., 1998).

Experimental Procedures

Trypanosome Culture and Transfection
Procyclic *T. brucei* strain 29.13, which coexpresses the Tet repressor protein and T7 RNA polymerase, was cultured and transfected as described in Wirtz et al. (1999).

Tandem Affinity Purification of Ligase Complexes
Generation of a TbREL1-TAP expressing *T. brucei* cell line was described previously (Panigrahi et al., 2003). Tagged TbREL1 complexes were purified from 2 l of cells as described (Panigrahi et al., 2003), with the following modifications. Eluates obtained after digestion with TEV protease (Invitrogen) were loaded onto 11 ml 10%–30% glycerol gradients and fractionated for 9 hr at 4°C and 38,000 rpm in an SW-40 rotor as described (Panigrahi et al., 2001a). In parallel gradients, catalase (232 kDa, 11S) and thyroglobulin (669 kDa, 19S) (Amersham) were run as size markers. 0.5 ml fractions were collected from the top. Selected fractions were pooled and purified over calmodulin resin (Stratagene) as described (Panigrahi et al., 2003). To generate a TbREL2-TAP expressing cell line, the complete coding sequence of TbREL2 was cloned into pLew79-TAP. Generation of recombinant cells and purification of TbREL2 complexes was performed as described above for TbREL1.

SDS-PAGE and Western Blotting
Cell lysates and protein samples were fractionated by SDS-PAGE, blotted, probed with mouse mAbs against TbMP81, TbMP63, TbREL1, and TbMP42, and developed using the ECL system (Amersham) as described (Panigrahi et al., 2001a). Polyclonal rabbit antisera against TbMP18 (N.L.E. et al., unpublished data) and TbMP57 (Ernst et al., 2003) were used at dilutions of 1:1000 and 1:10, respectively. Polyclonal rabbit antiserum against TbREL2 (a kind gift from S. Hajduk) was used at a dilution of 1:1000.

Adenylation Assays
Adenylation reactions were performed with 5 μl glycerol gradient fraction as described (Panigrahi et al., 2001a).

Mass Spectrometric Analysis
Samples were separated by SDS-PAGE and protein bands were visualized by silver staining. Protein bands were excised from the gel, digested with trypsin in-gel, and identified by tandem mass spectrometry as described (Panigrahi et al., 2001a).

In Vitro RNA Editing Assays
Precleaved insertion editing assays were performed as described (Igo et al., 2000) for 3 hr at 27°C in the presence of 100 μM UTP and 10 μM ATP. The reaction products were run on 11% polyacrylamide/7 M urea gels and visualized by phosphorimaging (Storm, Molecular Dynamics). Precleaved deletion editing assays were performed in the absence of UTP as described (Igo et al., 2002).

Yeast Two-Hybrid Analysis
Complete coding sequences for each of the proteins were amplified by PCR and cloned into the Gal4 AD vector pOAD and into the Gal4 BD vector pOBD2 (Uetz et al., 2000) by ligation or recombination cloning (Ma et al., 1987). pOAD and pOBD2 plasmids were introduced into haploid strains PJ69-4a and PJ69-4α (Uetz et al., 2000), respectively, using standard procedures. Empty plasmids as well as plasmids pOAD-PCL2, pOAD-PCL9, and pOBD2-PHO85 (Uetz et al., 2000) were included as negative and positive controls. Transformants were selected on solid medium lacking Leu and Trp, respectively. 96-well culture plates containing liquid YPAD medium were manually inoculated with pairs of pOAD and pOBD2 transformants to create a 27×26 array representing all combinations. Each gene fusion in the array was represented by a pool of several clones. Cultures were mated overnight at 30°C. Diploids were selected on OmniTray plates (Nunc) containing solid medium lacking Leu and Trp for 3–4 days at 30°C. For the two-hybrid selection, the diploids were transferred to medium lacking Leu, Trp, and His. To control the stringency of the screen, the medium was supplemented with 0-30 mM 3-AT. Colony transfers were performed manually or using a Beckman Biomek 2000 robot. Interactions were scored after 3–7 days of growth at 30°C, depending on the 3-AT concentration and the pair of proteins tested. Interactions were scored as positive when they emerged in at least two out of three screens, each time starting with haploids from separate transformations.

Coimmunoprecipitation Experiments
Plasmids for in vitro expression of TbMP63, TbREL1, and TbREL2 were described previously (Panigrahi et al., 2001b; Palazzo et al., 2003). The TbMP81 coding sequence minus the first 18 amino acids of the predicted preprotein plus a 6xHis tag was PCR-amplified from *T. brucei* genomic DNA and cloned into pSG1 vector (Schnaufer et al., 2001). ^{35}S-labeled proteins were expressed in a cell free, coupled transcription-translation system as specified by the manufacturer (Promega, TNT). Proteins were mixed and incubated for 10 min on ice. IgG-coated immunomagnetic beads (DYNAL) were coupled with specific antibodies as described (Panigrahi et al., 2001a). Protein combinations were incubated with antibody-coated beads overnight at 4°C with agitation. Washed precipitates were resuspended and physical interactions were visualized by SDS-PAGE and phosphorimaging.

RNA Ligase Assays
In vitro transcribed/translated proteins were immunoprecipitated individually as above and, individually or in combination, incubated with 50 fmol labeled 5′ mRNA fragment CL18, 1 pmol 3′ mRNA fragment CL13pp, 0.5 pmol gRNA gPCA6, and 0.3 mM ATP for 3 hr at 28°C and analyzed by denaturing PAGE and phosphorimaging as described (Igo et al., 2000).

Secondary Structure Analysis
Secondary structures for T7 DNA ligase and ChV ligase were taken from PDB files 1A0I and 1FVI, respectively, and aligned according to Odell et al. (2000) with minor manual adjustments. Secondary structures for TbREL1, TbREL2, TbMP81, TbMP63, TbMP42, and TbMP18 were predicted using the PROFsec program (http://cubic. bioc.columbia.edu/predictprotein/) and manually aligned to the structures of the T7 and ChV DNA ligases according to, in this order of priority, the conserved nucleotidyl transferase motifs, conserved primary sequences, and features of the secondary structures.

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