Kinetoplastid RNA-editing-associated protein 1 (REAP-1): a novel editing complex protein with repetitive domains

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Kinetoplastid RNA editing consists of the addition or deletion of uridines at specific sites within mitochondrial mRNAs. This unusual RNA processing event is catalyzed by a ribonucleoprotein (RNP) complex that includes editing site-specific endoribonuclease, RNA ligase and terminal uridylnucleotidyl transferase (Tutase) among its essential enzymatic activities. To identify the components of this RNP, monoclonal antibodies were raised against partially purified editing complexes. One antibody reacts with a mitochondrially located 45 kDa polypeptide (p45) which contains a conserved repetitive amino acid domain. p45 co-purifies with RNA ligase and Tutase in a large (~700 kDa) RNP, and anti-p45 antibody inhibits *in vitro* RNA editing. Thus, p45 is the first kinetoplastid RNA-editing-associated protein (REAP-1) that has been cloned and identified as a protein component of a functional editing complex.

Keywords: kinetoplastid RNA/REAP-1/ribonucleoprotein (RNP) complex/RNA editing

Introduction

Kinetoplastid RNA editing results in the addition or deletion of uridine residues at specific sites within several mitochondrial mRNAs (reviewed in Adler and Hajduk, 1994; Benne, 1994; Seiwert, 1995; Simpson and Thiemann, 1995). The specificity of this process, in both the site and number of uridines added or deleted, is dictated by small transcripts called guide RNAs (gRNAs). RNA editing is thought to be catalyzed by large ribonucleoprotein complexes (RNPs) containing gRNAs, pre-edited mRNAs and at least three essential enzymatic activities: editing site-specific endonuclease, terminal uridylnucleotidyl transferase (Tutase) and RNA ligase. A potential candidate for an editing complex has been identified previously by glycerol gradient sedimentation as a 35–40S RNP complex (Pollard *et al.*, 1992). This complex contains gRNAs, pre-edited mRNAs, an editing site-

specific endonuclease, Tutase and RNA ligase activities (Pollard *et al.*, 1992; Piller *et al.*, 1995; Sabatini and Hajduk, 1995; Corell *et al.*, 1996; Peris *et al.*, 1997). Glycerol gradient sedimentation also identified a 19S RNP complex. The 19S complex lacks pre-edited mRNAs but contains gRNAs, endonuclease, ligase and Tutase activities, and it can catalyze *in vitro* deletion editing (Corell *et al.*, 1996; Cruz-Reyes and Sollner-Webb, 1996; Seiwert *et al.*, 1996). A combination of the 19S and 35–40S complexes contains activity for uridine addition editing (Kable *et al.*, 1996). Purification of a ~20S complex that catalyzes U-deletion activity *in vitro* has been described (Rusché *et al.*, 1997). Surprisingly, the complex did not appear to contain gRNA.

At present, no component of a mature RNA-editing complex has been completely characterized or had its gene cloned. However, several candidate editing complex proteins have been identified. A gRNA-binding protein (gBP21) was purified and its gene cloned from *Trypanosoma brucei* (Koller *et al.*, 1997). However, gBP21 has not been shown to be a complex-associated protein, nor has its role in RNA editing been established. Another potential editing-associated activity, RNA helicase, has been identified in the mitochondrion of *T.brucei* (Missel and Goring, 1994). Helicase activity has been shown to co-sediment with RNA ligase and Tutase activities in the 35–40S fractions of glycerol gradients (Corell *et al.*, 1996). However, the role of RNA helicase in RNA editing has not been demonstrated. Several polypeptides, such as p51 (a homolog of mitochondrial aldehyde dehydrogenases) as well as hsp60 and hsp70 homologs and p18, subunit b of F₁F₀ ATPase (Speijer *et al.*, 1997), have been isolated from *Leishmania tarentolae* mitochondrial extract. On native gels these polypeptides co-migrate with specific RNP complexes that become labeled with [α -³²P]-UTP (Bringaud *et al.*, 1995); however, no direct experimental evidence exists to demonstrate the involvement of these polypeptides in RNA editing.

In this report, we describe the cloning and characterization of the first protein to be identified physically and functionally as a component of an RNA-editing complex. A monoclonal antibody against a 45 kDa protein found in partially purified 35–40S editing complexes was used to isolate the gene encoding a 45 kDa polypeptide that is mitochondrially localized in *T.brucei*. This 45 kDa protein co-fractionates with RNA ligase and Tutase as a component of a large (>700 kDa) RNP complex. Furthermore, anti-p45 antibody inhibits *in vitro* RNA editing. This RNA-editing-associated protein is termed REAP-1.

Results

Generation of monoclonal antibodies against complex-associated protein components

We have described previously the partial purification of two potential mitochondrial RNPs containing activities

Fig. 1. Western blot analysis shows distribution across glycerol gradient of proteins recognized by monoclonal antibodies. Proteins separated on a 10% SDS-PAGE gel were probed with eight different monoclonal antibodies raised against partially purified 35–40S complexes. The number at the top of each lane corresponds to the gradient fraction. The location of the 35–40S and 19S complexes in the gradient are indicated at the bottom. Proteins identified by each of the monoclonal antibodies are indicated by arrows. Molecular weight markers are shown to the left of the lanes.

involved in kinetoplastid RNA editing (Pollard *et al.*, 1992). In order to identify the individual components of these complexes, we generated a number of monoclonal antibodies against the 35–40S complex (see experimental procedures). Several of the antibodies were used to assay mitochondrial extract fractionated on glycerol gradients (Figure 1). Most of the monoclonal antibodies reacted with proteins present in both the 19S and 35–40S complexes. However, these proteins differ in their distribution across the glycerol gradient. For example, monoclonal 8c3 reacted with a 35 kDa polypeptide that distributes evenly throughout the gradient, whereas monoclonal 2c1 reacted with a 45 kDa polypeptide that enriches in the 35–40S complex-containing fractions. Western blot and Northern blot analysis of mitochondrial extracts fractionated by native gradient gel electrophoresis shows that the 2c1 reactive protein (p45) associates with a high-molecular-weight RNP containing both gRNA and mRNA (data not shown). Taken together, these results suggest that p45 might be a component of the editing complex.

Mitochondrial localization of p45

Since kinetoplastid RNA editing is exclusive to mitochondrial mRNAs, we expect p45 to localize to the mitochondrion of *T.brucei*. Immunofluorescence microscopy using the 2c1 monoclonal antibody shows the cellular localization of p45. The observed pattern of fluorescence suggests that p45 localizes to the cell's single mitochondrion (Figure 2). Co-localization of p45 with the trypanosomes respiratory chain protein, cytochrome *c*₁, further supports the mitochondrial localization of p45.

Anti-2c1 antibody screening of a cDNA expression library and cloning of p45

The 2c1 monoclonal antibody was used to screen a *T.brucei* expression library and a 675 bp cDNA was identified. The cDNA was subsequently used to identify a full-length clone from a genomic library. Southern blot analysis indicates that the p45 gene is nuclear and single copy (data not shown). The DNA sequence, shown in Figure 3, contains an open reading frame (ORF) encoding 418 amino acids with a calculated *M_r* of 45 343, which is consistent with the protein identified by the 2c1 antibody on Western blots. The putative AUG initiation codon is indicated and is ~50 bp downstream from two potential sites for *trans*-splicing of the 39-nt-spliced leader RNA common to the 5' end of all nuclear trypanosome mRNAs (Campbell *et al.*, 1984). Primer extension analysis has not distinguished which of the two is the primary site of *trans*-splicing (data not shown). Sequence comparison by BLAST analysis did not show significant homology to any protein in the current DDBJ/EMBL/GenBank database, nor were any sequence motifs recognized by the MOTIFS program from the Genetics Computer Group package. An interesting aspect of this 45 kDa protein is the presence of a 261-amino-acid repetitive domain. In order to sequence both strands of the complete cDNA a number of deletion clones were generated. Using these deletion clones, as well as the few third-position changes present, we are confident about the size of the repeat domain. This domain consists of a 21-amino-acid sequence tandemly repeated 7.7 times and is >90% identical at the DNA level (Figure 3). Thus, it appears that p45 is a unique protein with a highly conserved amino acid repeat.

To verify that the cDNA identified from the expression library corresponds to the same 45 kDa protein recognized by the 2c1 antibody we asked if the antibody would react with recombinant protein. Figure 4 shows Coomassie Blue-stained gels (A) and Western blot analysis (B) of recombinant protein expressed in *Escherichia coli*. The Western blot shows that before induction there is a small (<30 kDa) protein that cross-reacts with the monoclonal antibody to p45. Interestingly, in whole-cell lysates from either bloodstream or procyclic trypanosomes the antibody reacts only with the 45 kDa protein (data not shown). Three hours after induction in *E.coli* we see the appearance of an ~75 kDa band that represents glutathione *S*-transferase (GST)/p45 fusion protein. We also see the appearance of a number of smaller products that are recognized by the antibody. The cDNA for p45 contains a number of codons that are rarely found in *E.coli*. These rare codons probably cause premature termination resulting in the truncation products. This is further supported by our observation that the amount of the truncation products can be reduced either by supplying a plasmid that codes for a tRNA corresponding to one of the codons found frequently in p45 but infrequently in *E.coli*, or by generating third-position point mutations that change codons in p45 to those found more commonly in *E.coli*. In addition, these same truncation products are seen in the soluble material purified on GST affinity resin. After cleavage with thrombin to remove GST, the size of the largest band recognized by the antibody shifts down to the position of p45 and the truncation products also shift down accordingly. Taken together, the evidence shows that the cDNA

Fig. 2. Immunofluorescence microscopy of procyclic *T. brucei* shows that p45 is mitochondrially localized. Staining with DAPI (blue fluorescence) indicates the position of the nucleus and kinetoplast. Localization of p45 (green fluorescence) and cytochrome c_1 (red fluorescence) which is found in the mitochondrion. Regions of overlap (orange fluorescence) between the immunofluorescent signal for p45 and for cytochrome c_1 are shown. To the right of the panel a line drawing indicates the orientation of the trypanosome.

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1  AAAAAAGCTGATAGAGGGGAGAAATACGCCCAAGGGGCGCTCGTCA
51  AGTTCTCTCTGCTGGCGGCTCAGAACCCGACGTGGTTCAGTAG
101 CCGATTCTCTCTGCGATAGGCCATCAAGCGCCCGAAACGCTCTCCGA
151 TGGGACGCGGTAGGACTGGTGGCTGCTGCCACGCTTAAGATATCGTCA
201 ACACACACATACCGTCCGCGACGAGCAATGGATCCCTTAAACACAGC
251 AAAACACGCGACGGCGGAATGCCAAAAAATATCGCCACGAGGTCCGG
301 CAACCGCGAGTCTCGCGGAGTCAAGAGGTTAACATGTATCAAGG
351 GTGCCATCTACGACCAATGCCGACTTTTCCGAGGAAACCAACTGT
401 GGAGAAAACCGAAGCCCTCAGCCATTTGTCAGTGGGTTCGGAGAT
451 GTGCGCGGATATCTCAGATCATCGAAGCCCTCAGACACGAGTGA
501 CAGTCAAGAAGCGGTACGTGCGCGAGTCAGGACGTGAGGTACCGAAGC
551 GCATAAGACCACTCTCCAGGCGATCCAGTTCCCGAAATACACAGTG
601 CCGGAGGAGGTACGTGCGAGCAACAGCCACGAAACATCTACTATC
651 SGRQRRVPPVSAVDDVGSREKANNV
671 AGCGAAGCAACGAGTTCTGTAGTGCAGTAGATGAGGAAGCAGGAAAAGCTAACGTT
714 SERQRRVPPVSAVDDVGSREKANNV
734 AGCGAAGCAACGAGTTCTGTAGTGCAGTAGATGAGGAAGCAGGAAAAGCTAACGTT
777 SERQRRVPPVSAVDDVGSREKANNV
797 AGCGAAGCAACGAGTTCTGTAGTGCAGTAGATGAGGAAGCAGGAAAAGCTAACGTT
840 SERQRRVPPVSAVDDVGSREKANNV
860 AGCGAAGCAACGAGTTCTGTAGTGCAGTAGATGAGGAAGCAGGAAAAGCTAACGTT
903 SERQRRVPPVSAVDDVGSREKANNV
923 AGCGAAGCAACGAGTTCTGTAGTGCAGTAGATGAGGAAGCAGGAAAAGCTAACGTT
966 SERQRRVPPVSAVDDVGSREKANNV
986 AGCGAAGCAACGAGTTCTGTAGTGCAGTAGATGAGGAAGCAGGAAAAGCTAACGTT
1029 SERQRRVPPVSAVDDVGSREKANNV
1049 AGCGAAGCAACGAGTTCTGTAGTGCAGTAGATGAGGAAGCAGGAAAAGCTAACGTT
1092 AGCGAAGCAACGAGTTCTGTAGTGCAGTAGATGAGGAAGCAGGAAAAGCTAACGTT
1135 KAEQEDGRVTATISSDSR
1155 ATAAGAGCAAGAGACGGGCGAGTCACCGCAATTCTTCGACTCGAGA
1185 GCGCATTAAAGTACAGCAAGGCGAGTGGAGACAAAAAGTCTCGATCC
1235 L L T K D L I S H D A G R P K H
1255 GTTGTAAACAAAGATATAATTTCACGACGCGGCTGGTCTCCCAAC
1285 K S L N G L V I P Q V E G I K
1335 ATAAATCTCTTAATGGTGGTTAGTGATACCAAGGTGGAGGGGATTAAG
1355 S L L L F D I K P P K L S E T G A
1385 TCATTACTGCTTTTGTATCAAAACCCGAAACTGAGCGAGACTGGGC
1435 V A A T V
1455 TGTGTGCCGCACTGTGCGGTGAGAGCGTATGTGTCCATTACATCTTGGT
1485 ACGTGTCTTCCAAAGTGTAGCGTTTGTGTGAACCTTTGCATTCAGTTGG
1485 CGCAGAGCAGTTACGTTCTTCAATCAACAGGCAATTAATTTTGGTGAC
1535 ACTTGGCAGTGTGCAAGAGGGGAGGAGTGTGCAAAATCTCTGGA
1585 ACCTCCACGATAGTACCAATGAGAGCTTGAATTTTATTTTGGATAC
1635 GGTGGCAGTCTTCCGCGCTTCAACGAGCTGTGCGTATCATG

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Fig. 3. Sequence of novel p45 protein reveals a highly conserved 21-amino-acid repeat region. Genomic sequence and deduced amino acid sequence of p45. The gene codes for a protein of 418 amino acids with a predicted M_r of 45 343. The repeat region is boxed and repeats are aligned in order to show sequence conservation between repeats. Start and stop codons are shown in bold; two potential 5' splice sites are underlined; the 5' end of the partial cDNA is indicated by an arrow and the site of polyadenylation is overlined. Nucleotide and amino acid sequences are numbered on the left and right, respectively.

and genomic clones that have been identified code for the 45 kDa protein recognized by the 2c1 monoclonal antibody.

Co-purification of p45, RNA ligase and Tutsase in a large RNP complex

Sedimentation analysis and immunolocalization suggested that p45 might be an editing-complex protein. To investigate this possibility, we set out to determine whether p45 would co-purify in a RNP complex with RNA ligase and Tutsase activity. Preliminary studies with mitochondrial extracts from a related kinetoplastid, *Crithidia fasciculata*,

Fig. 4. Expression of recombinant p45 in *E. coli*. Recombinant p45 was expressed in *E. coli* as a C-terminal fusion with glutathione *S*-transferase (GST). (A) The left panel shows Coomassie Blue-stained gels of total protein before (uninduced) and 3 h after induction. The right panel shows the soluble protein purified on GST-affinity resin and the same sample after cleavage with thrombin to release p45. (B) Western blot analysis of samples shown in A. Arrows indicate the positions of full-length GST/p45 fusion and p45. Truncation products are marked with open circles.

indicated that RNA ligase and Tutsase activities co-purified as a gRNA containing RNP with ~10 other proteins (R.S. Sabatini, unpublished results). In addition, p45 was consistently found in the RNA-ligase-containing fractions. Therefore, a similar purification scheme was developed

Fig. 5. Co-fractionation of p45 and RNA ligase on ion exchange and affinity chromatography. Mitochondrial extract was fractionated sequentially on Q-Sepharose, Heparin-agarose and Phosphocellulose (P11). (A) Mitochondrial lysate was eluted from Q-Sepharose using a linear 100–800 mM NaCl gradient. Indicated fractions were adenylated and resolved on a 10% SDS-PAGE gel and autoradiographed (ligase panels), or separated on a 10% SDS-PAGE gel and analyzed by Western blotting with antibody to p45. Peak ligase fractions were further resolved on Heparin-agarose followed by phosphocellulose. The indicated fractions from each of the columns were treated as described above. S, start; F, flow-through. (B) A portion of each of the pooled fractions (see experimental procedures) from each purification step was separated on a 10% SDS-PAGE gel and silver stained: Start (crude mitochondrial lysate, 8 μ g), Q-Seph (Q-Sepharose, 7 μ g), Hep (Heparin-agarose, 16 μ g) and P11 (Phosphocellulose, 15 μ g). Sizes of the mol wt markers are indicated on the left. The two asterisks indicate the 50 and 57 kDa adenylated RNA ligases, and the arrow indicates p45.

for *T.brucei* based on following RNA ligase (Sabatini and Hajduk, 1995) which is easily identified by covalent adenylation with [α - 32 P]ATP. We then used Western blot analysis to ask whether p45 would be found in the RNA-ligase-containing fractions. As shown in Figure 5, p45 is consistently found in ligase-containing fractions through sequential Q-Sepharose, Heparin-agarose and phosphocellulose (P11) chromatography. After the Q-sepharose step the two proteins co-fractionate with the peak of adenylated ligase and of p45, both found in fraction 34. Following chromatography on heparin the peaks of RNA ligase and p45 are offset slightly, possibly indicating some heterogeneity within the editing complexes. It should be noted that heparin interferes with adenylation of the lower ligase. Subsequent further purification on P11 removes the heparin and restores the ability of the lower ligase to be adenylated. Chromatography on P11 results in a fairly sharp peak of adenylated ligase in fractions 10–12, and a slightly broader peak of p45 in fractions 8–14. We have previously seen on Western blots using

adenylated samples that p45 migrates to a position slightly below the lower adenylate ligase. Silver staining of the P11 fractions containing p45 and RNA ligase reveals 13–15 polypeptides, including p45 and the two adenylated RNA ligases (Figure 5B).

Further separation of this highly purified fraction by gel filtration chromatography resulted in two peaks of adenylated RNA ligase containing complexes ~700 and 450 kDa in size (Figure 6A and B). To determine whether these complexes contain gRNAs, the fractions with the peak RNA ligase were pooled and deproteinized, and the RNAs labeled with polynucleotide kinase. Labeled RNAs were then used as a hybridization probe. The probe hybridized to Southern blots of minicircle clones containing gRNA genes, weakly hybridized to maxicircle clones containing mRNA-coding genes and did not hybridize to maxicircle clones encoding mitochondrial rRNAs (Figure 6C).

Immunoblot analysis shows the peak of p45 to be centered on the larger side of the RNA ligase peak at

Fig. 6. p45 is present in RNP complexes containing activities essential for RNA editing. An aliquot of phosphocellulose-purified material (200 μ l) was adenylated with [α - 32 P]ATP and further purified on a Superose 6 column. (A) Adenylated RNA ligase localization across Superose 6 fractionation. The indicated fractions (500 μ l) were assayed for E-AMP by scintillation counting. The two peaks of adenylated ligase were compared with a separate run containing size standards. (B) p45, RNA ligase and Tutase cofractionate on gel filtration. Superose 6 fractions were acetone precipitated, separated on a 10% SDS-PAGE gel and immunoblotted with antibody to p45. An autoradiograph of the Western blot is shown to visualize adenylated ligases. Labeled products from Tutase assays were visualized by autoradiography. (C) gRNAs cofractionate with p45, RNA ligase and Tutase. RNA was isolated from pooled Superose 6 fractions corresponding to peak RNA ligase and labeled with [γ - 32 P]ATP, as described in Materials and methods. This was then used to probe dot blots of maxicircle clones containing gRNA sequences, maxicircle clones containing pre-edited mRNA sequences and maxicircle clones encoding mitochondrial rRNAs. pGEM is included as a vector control without insert. An autoradiograph is shown.

700 kDa (Figure 6B). Tutase activity also peaks slightly before RNA ligase, coincident with p45. These results suggest that p45 may be associated with a subset of RNA-ligase- and Tutase-containing RNP complexes. Following the final Superose 6 filtration step, the resulting complexes were estimated to be ~8000-fold purified based on Tutase activity (data not shown).

Co-immunopurification of p45 and RNA ligase

To determine directly whether p45 and RNA ligase were associated within the same ~700 kDa complex, we examined RNPs purified with an anti-p45 affinity column for RNA ligase. Purified RNPs were adenylated and Superose 6 fractions 21 and 22 were incubated with the anti-p45 column, extensively washed and eluted with high salt (≥ 2 M, see Materials and methods). Aliquots of the fractions corresponding to starting material, wash and eluate were analyzed on an SDS-PAGE gel. An autoradiograph of the gel indicates that both the 50 and 57 kDa ligases are present in the eluted material (Figure 7A, lane 3). To show that the co-immunopurification is specific for anti-p45 and not an artifact of the purification, the procedure was repeated with 19S and 35–40S RNPs from the glycerol gradient using an antibody affinity column against the iron-sulfur subunit of cytochrome *c* reductase

(Priest and Hajduk, 1994, 1995) (anti-FeS). As shown in Figure 7B, RNA ligase is specifically immunopurified from 'native' RNPs with anti-p45 but not with anti-FeS.

Antibody to p45 inhibits RNA editing *in vitro*

To investigate directly the role of p45 in RNA editing, we examined the effect of antibody against p45 on editing activity *in vitro* (Figure 8). The gRNA-dependent insertion of two uridines into labeled pre-edited A6 substrate RNA (Figure 8A) is inhibited by anti-p45 monoclonal antisera in a dose-dependent manner. Monoclonal antibodies to FeS (α -FeS) or to cytochrome *c* reductase complex subunit 2 (α -Sub 2) (Priest and Hajduk, 1994) had no significant effect on the *in vitro* reactions (Figure 8A and B). These results provide direct evidence for p45 being a component of an active editing complex.

Discussion

In this report we describe the identification and cloning of REAP-1, the first protein shown to be a component of the kinetoplast RNA-editing complex. The molecule is a 45 kDa polypeptide, 418 amino acids in length, which shows no homology to proteins in the DDBJ/EMBL/GenBank database. The *REAP-1* gene was cloned after

Fig. 7. Immunopurification of RNA ligase from the 700 kDa RNP with anti-p45. Fractions 21 and 22 from the Superose 6 column were bound and eluted from an anti-p45 column (see Materials and methods). (A) Direct visualization of the 50 and 57 kDa RNA ligase eluted from the anti-p45 (α -p45) column. Fractions corresponding to the starting material (with 1/10th of adenylated complex loaded), wash and eluted material were separated on a 10% SDS-PAGE gel and the adenylated RNA ligases visualized by autoradiography. (B) RNA ligase and p45 tightly associate with native 19S and 40S RNPs. Glycerol-gradient-purified 19 and 40S RNPs were adenylated and incubated with an anti-p45 (α -p45) and an anti-FeS (α -FeS) column. The columns were washed and protein was eluted as above. The peak radioactive fractions were acetone precipitated and separated on an SDS-PAGE gel, and the two adenylated RNA ligases visualized by autoradiography.

screening a cDNA expression library with an antibody generated against partially purified editing complex. The monoclonal antibody that recognized a 45 kDa polypeptide by Western blot analysis reacted with a partial cDNA clone which was subsequently used to clone a genomic fragment containing an open reading frame which encodes a 45 kDa protein. The sequence indicates a novel 261-amino-acid repetitive domain. This domain consists of a tandemly repeated 21-amino-acid sequence that is >90% identical at the DNA level. Immunofluorescence shows that REAP-1 localizes in the mitochondrion of procyclic forms of *T.brucei*. Immuno-electron microscopic studies further identify REAP-1 to be localized within the matrix of the mitochondrion (S.Madison-Antenucci and D.G. Russell, unpublished results). These results are consistent with REAP-1 representing a novel, nuclear-encoded 45 kDa polypeptide that is a soluble component of the mitochondrial matrix of *T.brucei*.

Both physical and functional data confirm that REAP-1 is a component of an active RNA-editing complex. REAP-1 co-purifies with both enzymatic activities and RNAs required for RNA editing. This purified RNP complex is stable up to at least 150 mM NaCl (R.S.Sabatini and S.L.Hajduk, unpublished results). Affinity chromatography followed by sizing shows that REAP-1, RNA ligase, Tutase and gRNA co-exist within a large (~700 kDa) RNP composed of ~13 polypeptides. Our finding that gRNAs are a component of this complex contrasts with a recent paper by Rusché *et al.* (1997), reporting that purified ~20S complexes do not contain gRNAs. However, our results are consistent with an earlier description (Pollard *et al.*, 1992) of 19S complexes identified on glycerol gradients. Based on size as well as the presence of Tutase, RNA

Fig. 8. Anti-p45 inhibits *in vitro* RNA editing. A6 U-addition *in vitro* editing reactions were performed with glycerol-gradient-purified extract. Reactions were incubated for 60 min with or without the addition of the indicated monoclonal antibodies. (A) *In vitro* editing is gRNA dependent and specifically inhibited by anti-p45. The panel on the left illustrates the gRNA dependence of the U-insertional editing reaction (Kable *et al.*, 1996). The arrow denotes the edited product with two uridines added. The reactions (+gRNA) were then repeated with increasing amounts of either anti-p45 (center) or anti-FeS (right) as a control antibody. In the presence of control antibody, variation in the amount of edited product was $\pm 5\%$. (B) Anti-p45 versus anti-FeS and anti-Sub 2. Percentage editing is plotted and is reported relative to the standard reaction (no antibody control). p45 and FeS data are a combination of two independent reactions. Key: anti-p45 (●), anti-FeS (▲) and anti-Sub 2 (○).

ligase and gRNA, the complex described here is likely to represent the 19S complex (Pollard *et al.*, 1992). The association of REAP-1 and RNA ligase in the same complex is further supported by the immunopurification of RNA ligase with anti-REAP-1. This association was also shown to exist in the partially purified 19S and 35–40S RNPs. Thus, the co-immunopurification of REAP-1 and RNA ligase from the ~700 kDa RNP is not an artifact of purification. Moreover, monoclonal antibodies against REAP-1 inhibit *in vitro* RNA editing in a dose-dependent manner.

In addition, the purification results suggest heterogeneity of the RNA-editing complexes. Specifically, two different sizes of RNPs which contain Tutase and RNA ligase appear to exist. They share characteristics of the complex I and complex II defined previously by glycerol gradient sedimentation of mitochondrial extract (Pollard *et al.*, 1992). Both complexes contain Tutase and RNA ligase, and the majority of REAP-1 associates with the larger

RNP. By glycerol gradient sedimentation, REAP-1 peaks in the 35–40S RNP and trails into the 20S RNP-containing fractions. Size exclusion chromatography, of highly purified editing complexes, confirms that REAP-1 is associated with the larger RNPs (~700 kDa). The distribution of components suggests heterogeneity of the RNP complex, with only a subset containing Tutase, RNA ligase and REAP-1. Given the relative size of the complexes containing all three components, these RNPs probably represent a mature form of the RNA-editing complex.

In further support of this heterogeneity, only 50% of the adenylylated purified complex bound to the anti-REAP-1 column. Whether this is indicative of the percentage of complexes containing REAP-1 or the limited accessibility of the protein in smaller complexes is unclear. The data clearly show that at least one form of the active editing complex contains REAP-1.

While several of the previously reported kinetoplastid mitochondrial proteins could contribute to the make-up of an RNA-editing complex, none has been directly shown to exist within functional editing complexes. This includes the recently identified *T. brucei* gRNA-binding protein (gBP21; Koller *et al.*, 1997) which may correspond to the ~25 kDa protein that is part of the ~13 polypeptide purified complex in this report. gBP21 was the first cloned and characterized protein potentially involved in kinetoplastid RNA editing. The protein was purified from crude *T. brucei* mitochondrial extract, and the recombinant protein shows specific gRNA-binding capabilities. A protein of similar size that binds gRNA, visualized by UV crosslinking, is present in the 19S complex from glycerol gradients (Corell *et al.*, 1996). The 19S complex is active for *in vitro* deletion editing (Corell *et al.*, 1996) and, as shown here, for *in vitro* addition editing. However, gBP21 has not been previously purified as a component of an RNP complex or has it been directly implicated in RNA editing.

Another enzyme, RNA helicase, has been proposed to be required in the RNA-editing reaction mechanism. The destruction of the gRNA:edited mRNA duplex by helicase may be required before the next gRNA:pre-edited mRNA duplex can form, so that editing can proceed along the mRNA. RNA helicase activity has been detected in the 35–40S mitochondrial complexes of *T. brucei* (Corell *et al.*, 1996). However, no direct evidence identifies it as a component of a functional editing complex.

Several proteins have been isolated from *Leishmania tarentolae* mitochondrial extracts as components of UTP-labeled complexes visualized by native gel electrophoresis (Bringaard *et al.*, 1995). Most of these, hsp60, hsp70, aldehyde dehydrogenase and subunit b of F_1F_0 ATPase (Speijer *et al.*, 1997), are homologous with proteins of known function outside of the editing process. What role these proteins would have in the editing mechanism remains unclear. While the data indicate that these polypeptides are contained in complexes, there is no evidence for them as components of editing complexes or for their involvement in the editing reaction.

In this report, we clone and identify REAP-1, a novel 45 kDa polypeptide, as a component of a purified (8000-fold) mitochondrial RNA-editing RNP complex. REAP-1 lacks homology with known proteins and its precise function in editing remains unknown. Its involvement in an active editing complex is shown by anti-REAP-1

antibody inhibition of *in vitro* editing. The identification of the first RNA-editing-complex-associated polypeptide, both physically and functionally, provides us with a powerful tool for elucidating further components of the editosome. The dissection of the RNP complex responsible for this unusual RNA processing event will, hopefully, enable us to understand both its origins and mechanism.

Materials and methods

Preparation of mitochondrial extracts

Mitochondria from exponentially growing *T. brucei* (TREU 667) were isolated as described previously (Harris *et al.*, 1990). Mitochondria from 2×10^{10} cells were resuspended in 1 ml of 25 mM Tris (pH 7.9), 50 mM KCl, 10 mM magnesium acetate, 1 mM EDTA, 1 mM dithiothreitol (DTT) and 5% glycerol. Mitochondria were lysed by the addition of 10% Triton X-100 to a final concentration of 0.5%. The lysate was gently mixed for 2 min at room temperature and subsequently clarified by centrifugation at 12 000 g and 4°C for 2 min. Supernatant was layered on the top of a 10–30% glycerol gradient (Pollard *et al.*, 1992). Following centrifugation, 16 fractions were collected from the bottom of the gradient, frozen on dry ice and stored at -70°C .

Antibody preparation

Female BALB/c mice were immunized twice, 4 weeks apart, by intraperitoneal injection with 1 mg and 0.1 mg of glycerol-gradient-purified 35–40S complex. For the first immunization, the complex was mixed with complete Freund's adjuvant. The subsequent immunization was accompanied by incomplete Freund's adjuvant. Four days after the second immunization the mice were sacrificed and the spleens were removed. Fusions were carried out by the U.A.B. Arthritis and Musculoskeletal Disease Center's Hybridoma Core Facility. Hybridoma cell lines were screened by ELISA and Western blot analysis. Hybridoma lines producing antibodies that are both ELISA and Western positive against the 35–40 RNP and its associated polypeptides were subcloned at least twice prior to ascites production.

Monoclonal antibody was purified from ascites fluid either by chromatography on protein A–Sepharose or DEAE–Sephacel (Pharmacia). The protein A column was washed with 10 column vol. of 100 mM Tris (pH 8.0) followed by 10 column vol. of 10 mM Tris (pH 8.0). Antibody was eluted with 0.5 M glycine (pH 3.0) and brought to neutral pH by the addition of 0.1 vol. 1.0 M Tris (pH 8.0). The DEAE column was prepared essentially as described by Bruck *et al.* (1982). The column was washed with five column volumes of 20 mM Tris (pH 8.0) and elution was performed in the same buffer with 50 mM NaCl.

Antibody column

Protein-A-purified monoclonal antibody against p45 was immobilized on a 2 ml Pierce Amino Link column according to the manufacturer's instructions. Five hundred microliters of adenylylated complex, Superose 6 fractions 21 and 22, was diluted 2-fold with phosphate-buffered saline (PBS) (150 mM NaCl) and incubated with the column, washed and protein eluted with 'gentle elution buffer' (Pierce) according to the manufacturer's instructions. Fractions (1 ml) were collected and adenylylated ligase assayed by scintillation counting of the entire fraction. Aliquots corresponding to starting material (1/10th of the loaded volume), wash (3 ml) and peak of adenylylated eluate (3 ml) were precipitated with 3 vol. of acetone, separated on a 10% SDS–PAGE gel and RNA-ligase visualized by autoradiography.

Investigating the co-immunopurification of p45 and RNA ligase from glycerol-gradient-purified RNPs, two antibody columns were prepared as described above using DEAE-purified antibodies against p45 and FeS. Two-fold more antibody was coupled to the p45 column versus FeS. Mitochondrial extract (1 ml) corresponding to the 19S and 40S fractions from glycerol gradient sedimentation, was adenylylated with 5 μCi [α - ^{32}P]ATP. Following dialysis (PBS, 150 mM NaCl), 500 μl was applied to each of the antibody columns and washed, and the protein was eluted as described above. The fractions corresponding to the peak of eluted radioactivity were pooled, acetone precipitated, separated on an SDS–PAGE gel, and adenylylated RNA ligase was visualized as described above.

Western blot analysis

Proteins were separated by SDS–polyacrylamide gel electrophoresis on 10% gels according to the method of Laemmli (1970) and transferred

electrophoretically onto nitrocellulose (Schleicher and Schuell BA85) at 4°C. Nitrocellulose blots were incubated at room temperature for 30 min in buffer containing 0.3% Tween-20, 20 mM Tris (pH 7.4), 0.5 M NaCl before the addition of the primary antibody (1:1000 dilution). Blots were developed using a 1:2000 dilution of biotin-labeled rabbit anti-mouse as a second antibody and a 1:500 dilution of alkaline phosphatase-labeled streptavidin.

Immunofluorescence microscopy

Log phase procyclic *T.brucei* were fixed in 8% EM-grade formaldehyde on ice for 1 h. The cell suspension was centrifuged, washed and resuspended in PBS. The cells were smeared onto microscope slides, air-dried, permeabilized with 0.1% Triton X-100 for 2 min and washed in PBS. The slides were then incubated with the p45 monoclonal antibody diluted 1:200 and the cytochrome *c*₁ antibody diluted 1:200. Slides were washed three times in PBS and incubated with secondary goat anti-mouse fluorescein-conjugated antibody and goat anti-rabbit biotin-conjugated antibody for 1 h at room temperature. Cells were washed three times in PBS and stained with Avidin/Texas Red diluted 1:20 for 1 h at room temperature. Cells were then washed and stained with 0.4 µg/ml DAPI in PBS and mounted in 0.1% phenylenediamine (Fisher) in PBS. Slides were then viewed with a digital fluorescence microscope.

RNA isolation and cDNA preparation

RNA was prepared from the procyclic form of *T.brucei* essentially as described (Chomczynski and Sacchi, 1987). Briefly, exponentially growing cells were collected by centrifugation at 6000 g and 4°C for 10 min, and washed once with an equal volume of PBS at 4°C. Cells were lysed at a concentration of 1×10^8 cells/ml in 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, 0.1 M β-mercaptoethanol. Nucleic acids were extracted by the sequential addition of 0.1 vol. 2 M sodium acetate (pH 4.0), 1 vol. phenol and 0.2 vol. of chloroform-isoamyl alcohol (49:1). Samples were kept on ice for 15 min before centrifugation at 10 000 g and 4°C for 20 min. RNA was precipitated from the aqueous phase by the addition of an equal volume of isopropanol. The pellet was resuspended in 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl and 0.1 M β-mercaptoethanol, and precipitated again from isopropanol. The pellet was then resuspended in 10 mM Tris (pH 7.5), 1 mM EDTA, extracted with an equal volume of phenol followed by phenol-chloroform (1:1) and precipitated from ethanol with 0.3 M sodium acetate (pH 4.5). Poly(A) RNA was purified from total RNA using Oligotex resin, as suggested by the supplier (Qiagen).

First-strand cDNA synthesis was performed using an oligonucleotide containing a *NotI* restriction site. Following second-strand synthesis, the cDNA was treated with T4 DNA polymerase to ensure blunt ends and then size-selected on a Sepharose CL-4B column as described (Ausubel *et al.*, 1989). Size-selected cDNA was ligated with *EcoRI* adapters, treated with T4 polynucleotide kinase, and digested with *NotI* endonuclease. Excess adapters were removed by passage over a second Sepharose CL-4B column. In order to generate a directionally cloned library the resulting cDNA was ligated with λgt11 vector that had been digested with *EcoRI* and *NotI*.

cDNA and genomic cloning

Immunological screening of the procyclic expression library was performed as described previously (Sambrook *et al.*, 1989) using a 1:1000 dilution of ascites fluid containing monoclonal antibody 2c1. Filters were developed essentially as described above for Western blots. Incubations were at 4°C in buffer containing 0.3% Tween-20, 20 mM Tris (pH 7.4), 0.15 M NaCl. Isolation and rescreening of potentially positive plaques yielded a partial cDNA clone. The cDNA insert was subcloned into Bluescript SK+ and sequenced according to the manufacturer's instructions (United States Biochemical). Based on the sequence obtained, two primers (upstream; 5'-GGACTCGAGAGGCGCATTAAGTC-3' and downstream; 5'-CAGACTCACTGCCAACTGTCCACC-3') were used to generate a 376 bp PCR product. In order to obtain a clone containing the entire coding region, the PCR product was used to screen a genomic library. A 7 kb clone was isolated and subjected to *ExoIII* digestion in order to generate a series of deletion clones that could be sequenced using a primer corresponding to the Bluescript SK+ vector.

Purification of editing complexes

The editing complex was purified from 100 l of *T.brucei* (110×10^9 cells) by following the purification of RNA ligase. Mitochondria were isolated as described previously (Pollard *et al.*, 1992). Extract was

prepared by lysis of the mitochondria with 0.5% CHAPS in buffer A (50 mM Tris 7.9, 0.1 mM EDTA, 100 mM NaCl, 10% glycerol), containing the protease inhibitors PMSF (10 µM), leupeptin (10 µg/ml) and pepstatin (10 µg/ml). Debris from the mitochondrial lysate was removed by sedimentation (12 000 g, 10 min, 4°C). The mitochondrial lysate was diluted 2-fold with buffer A (without NaCl), and treated in a batch with Q-Sepharose (Pharmacia, 50 ml packed vol) at 4°C for 1 h. The resin was transferred to a column (5×10 cm) and washed with buffer A (with 100 mM NaCl), and protein was eluted using a 200 ml linear gradient from 100–800 mM NaCl. RNA ligase eluted at about 400 mM. The RNA-ligase-containing fractions were then pooled and dialyzed against buffer A (50 mM NaCl) and then loaded onto a 30 ml Heparin column (BioRad; 2.5×8 cm) at 1 ml/min. The column was washed with four column vol. of buffer A (150 mM NaCl, 10 µM PMSF, 5 µg/ml leupeptin, 5 µg/ml pepstatin). RNA ligase was eluted at 1 ml/min using a 150 ml linear gradient from 150–500 mM NaCl. The active fractions (eluting between 250 and 350 mM NaCl) were pooled, dialyzed as before and loaded onto an 8 ml phosphocellulose column (Whatman; 1.5×8.5 cm) at 1 ml/min. The column was washed with four column vol. of buffer A (100 mM NaCl) followed by a linear 300–600 mM NaCl gradient. The active fractions were pooled and aliquots (200 µl) further purified by gel filtration on a 30 ml Superose 6 FPLC column (Pharmacia) at 0.25 ml/min with buffer A (100 mM NaCl, 10 µM PMSF, 2 µg/ml leupeptin and 2 µg/ml pepstatin). In most cases, the fraction was adenylated prior to gel filtration with the addition of 5 µCi of [α-³²P]ATP (and 10 mM final concentration MgCl). Fractions of 500 µl were then collected and assayed directly for adenylated ligase (E-AMP) by scintillation counting the entire fraction.

gRNA hybridizations

Superose 6 fractions corresponding to the peak RNA ligase activity were pooled and incubated with SDS (0.5%) and proteinase K (100 µg/ml) at 25°C for 10 min. Following extraction with phenol-chloroform and chloroform, the nucleic acids were precipitated in ethanol. The nucleic acids were then bacterial-alkaline-phosphatase treated and labeled with T4 polynucleotide kinase and [γ-³²P]ATP. The labeled nucleic acids were identified as RNase A sensitive and used as a hybridization probe. This probe was used to hybridize a series of dot blots representing minicircle and maxicircle sequences. Minicircle clones were generated from a Taq digestion of *T.brucei* kDNA as described by Pollard and Hajduk (1991). Maxicircle clones, corresponding to Cyb, MURF1, ND1 and CoII sequences, were generated as described by Michelotti and Hajduk (1997). A clone representing 2.2 kb of the mitochondrial rRNA genes as well as pGEM vector are included as controls. A total of 5 µg of DNA were blotted onto 0.05-µm nitrocellulose (Schleicher and Schuell) as described by Bio-Rad (Bio-Dot microfiltration apparatus). The filters were pre-hybridized in a solution of 1 M NaCl₂, 10% dextran sulfate and 1% SDS for 3 h. Salmon sperm DNA (100 µg/ml) and kinase-labeled RNA (isolated from the purified complex as described above) were added and hybridization was allowed to proceed for 12 h. Following hybridization, the filters were washed in 1× SSC at 55°C.

Enzyme assays

E-AMP formation and Tase assays were performed as described previously (Sabatini and Hajduk, 1995). *In vitro* editing reactions were performed as described by Kable *et al.* (1996), but without the addition of CaCl₂ or yeast RNA. Antibody inhibition assays utilized active fractions (19S) from glycerol-gradient-purified mitochondrial extract (see above). Monoclonal antibody (Protein A purified), at the noted concentration, was added to 10 µl of extract prior to the addition of substrate. Relative percentage editing is expressed to control for degradation of substrate by non-specific RNase activity in the antibody preparation. All reactions are compared to the no-antibody control, which was taken to give 100% editing. No-antibody control reactions typically resulted in 2–3% conversion of substrate to edited product.

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