Endonuclease Associations with Three Distinct Editosomes in Trypanosoma brucei

Three distinct editosomes, typified by mutually exclusive KREN1, KREN2, or KREN3 endonucleases, are essential for mitochondrial RNA editing in Trypanosoma brucei. The three editosomes differ in substrate endoribonucleolytic cleavage specificity, which may reflect the vast number of editing sites that need insertion or deletion of uridine nucleotides (Us). Each editosome requires the single RNase III domain in each endonuclease for catalysis. Studies reported here show that the editing endonucleases do not form homodimeric domains, and may therefore function as intermolecular heterodimers, perhaps with KREPB4 and/or KREPB5. Editosomes isolated via TAP tag fused to KREPB6, KREPB7, or KREPB8 have a common set of 12 proteins. In addition, KREN3 is only found in KREPB6 editosomes, KREN2 is only found in KREPB7 editosomes, and KREN1 is only found in KREPB8 editosomes. These are the same associations previously found in editosomes isolated via the TAP-tagged endonucleases KREN1, KREN2, or KREN3. Furthermore, TAP-tagged KREPB6, KREPB7, and KREPB8 complexes isolated from cells in which expression of their respective endonuclease were knocked down were disrupted and lacked the heterotrimERIC insertion subcomplex (KRET2, KREPA1, and KREL2). These results and published data suggest that KREPB6, KREPB7, and KREPB8 associate with the deletion subcomplex, whereas the KREN1, KREN2, and KREN3 endonucleases associate with the insertion subcomplex.

Transcripts from most mitochondrial genes in Trypanosoma brucei require post-transcriptional RNA editing, in which uridine nucleotides (Us) are inserted or deleted to generate translatable mRNAs (1). The mitochondrial transcriptome includes thousands of guide RNAs (gRNAs) that are used as templates to recode mRNAs (2). Protein complexes called editosomes recognize RNA substrates and coordinate the catalytic steps of editing. Three distinct ~20 S editosomes, first identified by tandem affinity purification (TAP) tag fused to endonucleases KREN1, KREN2, or KREN3, can cleave mRNA, insert or delete Us, and ligate mRNA fragments back together (3, 4). These editosomes have 12 proteins in common, and sets of mutually exclusive proteins: KREN1/KREPB8/KREX1, KREN2/KREPB7, or KREN3/KREPB6 (Fig. 1A). Although these ~20 S editosomes contain all the necessary catalytic activities of RNA editing, mitochondrial RNA processing ultimately involves multiple protein complexes.

Dissecting interactions between and within the numerous complexes involved in editing has proved to be a challenging task that is far from completion. A growing list of proteins and complexes (MRP1/2, MRB1, RBP16, REAP1, TbRGG1, TbRGG2, KRET1, MEAT1, KPAP1) have functions that affect RNA editing by modulating mRNA/gRNA binding, altering RNA stability, or functioning in ways that are still being elucidated. For example, in vitro RNA editing activity is enhanced by either MRP1/2 or RBP16, presumably reflecting in vivo interactions with editosomes (20, 21). Recently, an extensive network of interactions among editosome proteins was revealed by a combination of yeast two-hybrid analysis and subcomplex reconstruction with recombinant proteins (22). However, interactions were only identified among the common set of 12 editosome proteins in these experiments, leaving interactions involving the editing endonucleases and their uniquely associating proteins unresolved. Within the common set of 12 proteins are two heterotrimeric subcomplexes: KREX2-KREPA2-KREL1 and KRET2-KREPA1-KREL2, which are capable of deletion and insertion editing, respectively, on pre-cleaved substrates in vitro (23). The separate deletion and insertion heterotrimeric subcomplexes are “bridged” by KREPA3 and KREPA6 in the intact editosome (22). Understanding endonuclease interactions with other editosome proteins may be critical to understanding the nature of differential substrate recognition by each endonuclease, as well as the roles played by KREPB6, KREPB7, and KREPB8.

Each editing endonuclease, and therefore editosome, has distinct substrate specificity. KREN1 editosomes primarily cleave at deletion editing sites, KREN2 editosomes primarily cleave at most insertion editing sites, and KREN3 editosomes primarily cleave at COII insertion editing sites (3, 24, 25). All three editing endonucleases are essential, and each has a single conserved RNase III motif that is required for cleavage activity (26). All characterized RNase III endonucleases function as dimers: intermolecular homodimers such as archetypal Escherichia coli RNase III, or intramolecular heterodimers such as Dicer. Each subunit of the RNase III dimer is responsible for cleaving one
RNA strand, so that both strands of double-stranded RNA substrate are typically cleaved (26). Editosomes contain two proteins with degenerate RNase III motifs, KREPB4 and KREPB5, that have been proposed to function as intermolecular heterodimers with KREN1, KREN2, and/or KREN3 (3). Although KREPB4 and KREPB5 are essential and required for integrity of the editosome, physical and/or functional interactions with the endonucleases have not yet been demonstrated (27, 28). The degeneracy of KREPB4 and KREPB5 RNase III motifs suggests that they would not be catalytically active and this is supported by limited mutational analysis.3 Hence, such intermolecular heterodimers would be predicted to cleave only one strand of RNA. This proposal is attractive, because it predicts that the mRNA would be cleaved and gRNA would not, permitting gRNA recycling. However, experimental evidence for such intermolecular heterodimerization is lacking.

The functional roles of the proteins that uniquely associate with each type of endonuclease are incompletely understood. Because KREN1 editosomes primarily cleave at deletion editing sites, the unique presence of KREX1 is consistent with its characterization as a U-specific exoribonuclease (29, 30). In contrast, the functional roles of KREPB6, KREPB7, and KREPB8 are unknown (1, 4). The sequence similarity of KREPB6, KREPB7, and KREPB8, which includes a U1 zinc finger motif, implies that these proteins act in similar functional roles, and their exclusive presence with respective endonucleases suggests a role associated with the cleavage of mRNA. The editing endonucleases also have U1 zinc finger motifs, prompting speculation that these domains function in ways analogous to the archetypical zinc finger in spliceosomal U1C protein from U1 small nuclear ribonucleoprotein particle (U1 snRNP). The U1 snRNP plays a critical role in the initial stages of splicing by recognizing the 5’ splice site and directing the subsequent assembly of the functional spliceosome. U1C promotes base pairing of the 5’ end of U1 snRNA to the 5’ splice site, and interaction of U1C with U1 snRNA requires other proteins from the U1 snRNP (31, 32). The functional evidence from the U1C zinc finger, therefore, suggests that an analogous function in RNA editing might be the recognition of mRNA/gRNA duplexes, and perhaps editing sites, in the context of other editosome proteins at the initial step of endonucleolytic cleavage.

In this paper we examine protein associations within the three distinct editosomes and show that editosomes purified via TAP tag fused to KREPB6, KREPB7, or KREPB8 mirror those previously isolated via tagged KREN3, KREN2, or KREN1, respectively. We also show that KREPB6, KREPB7, and KREPB8 preferentially interact with the portion of the editosome that contains the deletion subcomplex in the absence of endonuclease expression. Finally, we provide the first experimental evidence for intermolecular heterodimerization of RNase III endonucleases.

**EXPERIMENTAL PROCEDURES**

*Plasmid Construction and Transfections—*To create cell lines expressing C-terminal TAP-tagged proteins under tetracycline regulation, coding sequences were PCR amplified from *T. brusa* cei strain 427 genomic DNA and cloned into the pLEW-MHTAP plasmid (33). Oligos for PCR amplification were ATAAAGCTTATGACCTCTGTATTTGGAT (5571) and AAGGATCCCTCTGAAAGACATCTCT (5572) for KREPB6; ATAAAGCTTATGTTTCTAAAAACCCACTTGC (5573) and CGAGGATCCCACTCTTTAGAGGTGATGAGTTGAG (5574) for KREPB7; ATAAAGCTTATGCAACCGGCTAGACGGCCGG (5602) and AAGGATCCCAATATCTCATGCTGCAA (5603) for KREPB8. PCR amplicons for each coding sequence were digested with HindIII and BamHI and cloned into the same sites in pLEW-MHTAP to make pKREPB6-TAP, pKREPB7-TAP, and pKREPB8-TAP plasmids. Plasmids were digested with NotI and transfected into PF 29.13 cells, which co-express tet repressor and T7 RNA polymerase; KREPB6-TAP, KREPB7-TAP, and KREPB8-TAP cell lines were isolated via puromycin selection. Each cell line was tested to confirm tetracycline-dependent expression (data not shown).

To create cell lines that constitutively express these TAP-tagged proteins, coding sequences were PCR amplified using oligos 5571, 5573, or 5602 with oligo 6063 (ATTCATGATCAGGTTGAATCTCCCCCGCGGAATTC). Coding sequences with NotI-digested plasmids were then transfected into existing PF 29.13 lines that express the TAP-tagged version of that endonuclease, HindIII and BamHI flanked coding sequences for KREN1, KREN2, and KREN3 were cloned into the same sites in p Lew79–3V5(PAC) (34). NotI-digested plasmids were then transfected into existing PF 29.13-derived lines that express the TAP-tagged version of each endonuclease (4). Cell lines KREN1-TAP, KREN1V5, KREN2-TAP, KREN2V5, and KREN3-TAP, KREN3-V5 were isolated by puromycin selection. To create cell lines that co-express both TAP-tagged and V5-epitope-tagged versions of editing endonucleases, HindIII and BamHI flanked coding sequences for KREN1, KREN2, and KREN3 were cloned into the same sites in pLew79–3V5(PAC) (34). NotI-digested plasmids were then transfected into existing PF 29.13-derived lines that express the TAP-tagged version of each endonuclease (4). Cell lines KREN1-TAP, KREN1V5, KREN2-TAP, KREN2V5, and KREN3-TAP, KREN3-V5 were isolated by puromycin selection.

**TAP Tag Purifications**—Expression of TAP-tagged proteins in PF KREPB6-TAP, KREPB7-TAP, and KREPB8-TAP cell lines was induced for 48–72 h with 200–500 ng/ml of tetracycline, and 1–2 liters of cells were harvested at ∼2 × 10^7 cells/ml. Crude mitochondrial preparations were made from harvested cells, lysed in 20 ml of IPP150, 1% Triton X-100, Complete protease inhibitors at 4 °C, and subsequently clarified by centrifugation at 10,000 × g. Tagged complexes were purified from clarified mitochondrial lysates by sequential IgG and calmodulin affinity chromatography (35). In parallel, 10–30% glycerol gradient fractionation of TEV eluates was performed as previously described (23) except for a longer (12 h) fractionation at 38,000 rpm at 4 °C in SW-40 rotor (Beckman). For BF cell lines KRO-KREN3-KREPB6-TAP, KRO-KREN2-KREPB7-TAP, and KRO-KREN1-KREPB8-TAP, expression of the TAP-tagged protein is constitutive, whereas expression of the endonuclease was regulated by tetracycline. To modulate endonuclease expression, cells were centrifuged at 1,300 × g for 10 min, resuspended in medium lacking tetracycline, then
recentrifuged and resuspended again. Cells were then split into medium with (expressed) or without (repressed) tetracycline. After 72 h of growth, cells at ~2 × 10^6 cells/ml were harvested for all cell lines, 2 liters each with and without tet for RKO-KREN3-KREPB6-TAP and RKO-KREN2-KREPB7-TAP; 6 liters each with and without tet for RKO-KREN1-KREPB8-TAP. To harvest, cells were centrifuged at 1,300 × g for 10 min, resuspended in 30 ml of medium, recentrifuged, and cell pellets were flash frozen on liquid nitrogen. Frozen cells were resuspended in 20 ml of IPP150, 1% Triton X-100, Complete protease inhibitors (Roche Applied Science) at 4 °C, and processed as above.

**SDS-PAGE and Western Analyses**—For direct visualization of protein complexes, samples were resuspended in SDS-PAGE loading buffer and resolved on 10% SDS-PAGE gels (Criterion Tris-HCl, Bio-Rad). Gels were stained with SYPRO Ruby using the manufacturer’s protocol (Molecular Probes), and bands were visualized using Alpha Innotech Alphalmager EP. For Western analyses in Figs. 2 and 7 as well as supplemental Fig. S1, purified protein complexes were resolved on Criterion 10% SDS-PAGE gels, transferred to Millipore Immobilon-FL membranes (LiCor), and blocked overnight at 4 °C in Odyssey blocking buffer. PageRuler ladder (Fermentas) was used as a size reference on each blot. Blots were simultaneously probed in Odyssey blocking buffer (LiCor) with mouse monoclonal antibodies against KREPA1, KREPA2, KREL1, and KREPA3 (36) and either 1:10,000 rabbit polyclonal antibody against the calmodulin binding peptide (GenScript) (for Fig. 2) or 1:10,000 rabbit polyclonal antibody against the V5 epitope (GenScript) (for Fig. 7) in Odyssey blocking buffer. After 4 washes with 1× PBS-T, blots were probed with 1:15,000 IRDye 680-conjugated goat anti-rabbit (LiCor) and IRDye 800-conjugated goat antimouse (Rockland) secondary antibodies. Blots were washed 4 times with 1× PBS-T and 3 times with 1× PBS, dried, and scanned with a LiCor Odyssey scanner, and analyzed with Odyssey version 3.0 software. For Western analyses in Fig. 5 and supplemental Fig. S2, blots were developed with the ECL kit (Pierce) per the manufacturer’s instructions.

**Mass Spectrometry**—Proteins in editosome samples isolated by tagged KREPB6, KREPB7, and KREPB8 were denatured with 8 M urea, diluted 1:8, and the proteins were digested in solution with trypsin, and the resulting peptides were fractionated and analyzed by tandem mass spectrometry (LC-MS/MS) as described (36, 37). Identified peptides corresponding to editosome proteins are indicated in supplemental Table S2.

**Adenylation Assays**—Autoadenylation of KREL1 and KREL2 with [α-32P]ATP was performed as previously described (38). Proteins were resolved on 10% Criterion (Bio-Rad) SDS-PAGE gels that were then fixed in 50% methanol, 10% acetic acid, equilibrated in 10% methanol, 4% glycerol, dried, and analyzed by PhosphorImager (GE Healthcare).

**Editing Assays**—Pre-cleaved editing assays were performed as previously described: for deletion, RNAs were radiolabeled U5–5′CL with U5–3′CL and gA6[14]PC-del (39); for insertion, RNAs were radiolabeled 5′CL18 with 3′CL13pp and gPCA6–2A (40). Reaction products were detected by polyacrylamide-urea gel electrophoresis and phosphorimaging. Full round in vitro insertion (41) and deletion (42) editing assays were modified to optimize for the cleavage product as previously described (3) using radiolabeled A6-eES1 pre-mRNA with gA6[14] gRNA and radiolabeled A6short/TAG1 pre-mRNA with D34 gRNA, respectively. Trio site substrate (GGGCAUUAAUAGUUCCGUGGUGUAAUGAAAAGGAAGGGGAAAGGUAUAUAAUGAAAAGGGGAUUUAAAG) was derived from COIIcisU1, and assayed as previously described for that substrate for Fig. 4D (3). For Fig. 4E, 1 μM ADP was added to all assays to stimulate the deletion cleavage activity. Each editing assay used 15 μl of sample.

**Complex Purification from Cell Lines Co-expressing Tagged Endonucleases**—Cell lines (KREN1-V5, KREN1_V5, KREN2-V5, KREN2_V5, KREN3_V5, TAP-KREN1, TAP-KREN2, and TAP-KREN3) were grown in the presence of 200 ng/ml of tetracycline for 48–72 h, and harvested at ~2 × 10^7 cells/ml. Aliquots of ~2 × 10^5 total cells were set aside for immunoprecipitations. For cell lines expressing V5-tagged endonuclease, TAP-tagged complexes were isolated from the remaining (~4 × 10^10) cells as described above. For samples from parental TAP-KREN1, TAP-KREN2, and TAP-KREN3 cell lines, calmodulin-eluated fractions 2–4 were pooled and 70 μl combined with 30 μl of 2× SDS sample buffer, and 20–25 μl were used for Western blot. For KREN1-V5, KREN1_V5, KREN2-V5, KREN2_V5, and KREN3_V5 cell lines, calmodulin-eluated fractions 2–4 were pooled and 680 μl incubated with 30 μl of StrataClean resin (Stratagene) for 10 min on ice to concentrate samples. Resin was spun for 2 min at 2,000 × g, supernatant was discarded, complexes were resuspended in 65 μl of 2× SDS sample buffer, and 8 μl used for Western analysis.

**Immunoprecipitations**—For each immunoprecipitation, 30 μl of goat anti-mouse IgG Dynabeads (Invitrogen/DYNAL) were washed twice with 2 ml of 1× PBS, 0.1% BSA and incubated with 0.5 μg of Protein A (Sigma) to minimize nonspecific binding of the TAP-tagged endonucleases to the Fc portion of bound IgG. Beads were then incubated with 500 μl of 1× PBS, 1% BSA and 500 μl of mouse antibody specific for KREPA2 (36), 500 μl of mouse antibody (mAb 78) specific for heat shock protein 70 (43), or 0.5 μl of mouse antibody specific for the V5 epitope tag (Invitrogen) for 2 h of rotating at 4 °C. Beads were then washed four times with 1 ml of cold IPP150 (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40) and incubated for 2 h rotating at 4 °C with cleared lysate (5 × 10^5 cells lysed in IPP150 with 1% Triton X-100, followed by centrifugation at 10,000 × g at 4 °C). After incubation, the supernatant was removed, and beads were washed four times with 1 ml of IPP150. Complexes bound to beads were eluted with 100 μl of 2× SDS sample buffer, heated for 5 min at 95 °C, and 12 μl were used for Western blot analysis. Sample volumes (as stated above) were adjusted to yield similar intensities of KREPA1, KREPA2, KREL1, and KREPA3 signals.

**RESULTS**

**Analysis of TAP Tag Isolated KREPB6, KREPB7, and KREPB8 Complexes**—TAP tags (4, 35) were added to the C terminus of KREPB6, KREPB7, or KREPB8, and subsequently expressed in PF 29.13 cells (Fig. 1B). Growth of cells was unchanged by tetracycline induction of expression of the TAP-tagged protein, and was indistinguishable from parental cell lines (data not
shown). Two days after expression of the TAP-tagged protein induced by tetracycline the cells were harvested and crude mitochondria were prepared. TAP-tagged complexes were sequentially affinity purified from lysed crude mitochondria by IgG-Sepharose and calmodulin affinity columns as previously described (4). Western analysis of mitochondrial lysates using recombinant peroxidase anti-peroxidase, which binds to the Protein A moiety of the TAP tag, showed roughly equivalent expression of the tagged proteins in vivo (data not shown).

Protein complexes isolated via TAP tag on KREPB6, KREPB7, and KREPB8 were visualized by resolving calmodulin eluates on 10% SDS-PAGE gels and staining with SYPRO Ruby (Fig. 2A). Although the overall profile of these protein complexes was similar, certain bands were unique to particular tagged proteins. KREPB6 complexes have a band at ~67 kDa predicted to be KREN3, KREPB7 complexes have a band at ~61 kDa predicted to be KREN2, and KREPB8 complexes have a band at ~100 kDa predicted to be KREX1. Although KREN1 is uniquely present in KREPB8 complexes, at this resolution it overlaps with KREX2, which is present in all complexes (data not shown). Bands corresponding to the tagged protein for KREPB6 (~57 kDa after TEV cleavage) and KREPB7 (~55 kDa after TEV cleavage) also appear to overlap with a shared editosome protein, KRET2. Tagged KREPB8 (~50 kDa after TEV cleavage) appears to migrate closer to ~45 kDa. Overall the complexes have very similar composition, but few distinct proteins.

TAP-isolated complexes were further analyzed by Western blot and adenylation assays. A 20 S glycerol gradient fraction of lysed, purified mitochondria serves a positive control. Western analysis of KREPB6, KREPB7, and KREPB8 complexes using antibodies against the common set of editosome proteins KREPA1, KREPA2, KREL1, and KREPA3 shows that all four proteins are present in each sample, although it appears that KREPB8 complexes have a lower amount of KREPA1 and KREPA3 signals relative to the KREPA2 and KREL1 signals (Fig. 2B, top). Simultaneous probing for KREX1 reveals robust signals for KREPB8 and 20 S mitochondrial control and much weaker signals for KREPB7 and KREPB6 (Fig. 2B, middle). Quantitation of the KREX1 signal intensity normalized to the total signal for the set of common editosome proteins KREPA1, KREPA2, KREL1, and KREPA3 reveals that KREPB6 and KREPB7 have 66.1- and 36.0-fold less KREX1, respectively, compared with KREPB8, whereas the 20 S control has 1.3-fold less. In contrast, among editosomes isolated via TAP-tagged endonuclease, only KREN1 has a detectable KREX1 signal (Fig. 2D). Western analysis of KREPB6, KREPB7, and KREPB8 complexes showed the presence of the tagged proteins in the eluates.
plexes using antibody against the calmodulin-binding peptide (aCBP) detects the tagged protein, and shows single bands for KREPB6 and KREPB7 that have similar apparent sizes, whereas multiple bands for KREPB8 suggest a predominant intact protein with minor degradation products below (Fig. 2B, bottom). Adenylation assays covalently radiolabel editing ligases KREL1 and KREL2, and reveal that KREPB6, KREPB7, and KREPB8 complexes have significantly more signal for KREL1 than KREL2 in comparison to 20 S mitochondrial control (Fig. 2C). These results show that the three editosomes have substantial similarity.

Tandem mass spectrometry was used to identify editosome components in the isolated in KREPB6, KREPB7, and KREPB8 complexes. Although each complex contains a common set of editosome proteins, KREN3 was only found with tagged KREPB6, KREN2 was only found with tagged KREPB7, and KREN1 and KREX1 were only found with tagged KREPB8 (Table 1). The identification of mutually exclusive editosome proteins matches previous results obtained from analyzing TAP-isolated KREN1, KREN2, and KREN3 editosomes (3, 4). Together, these results indicate that TAP-isolated KREPB6, KREPB7, and KREPB8 complexes contain editosomes similar to, if not the same as, those isolated via TAP-tagged KREN3, KREN2, and KREN1, respectively. Thus, distinct compositional differences between these three editosomes distinguish substantially similar complexes. Analysis of editing activities in KREPB6, KREPB7, and KREPB8 editosomes reveal distinct endonucleolytic cleavage specificities for each editosome. Pre-cleaved insertion and deletion assays were performed to assess whether each complex had the editing activities downstream of endonucleolytic cleavage (Fig. 3). KREPB6, KREPB7, and KREPB8 editosomes were all able to specifically add Us and ligate RNA (Fig. 3B). In contrast, only KREPB7 editosomes cleave standard insertion-site substrate based on ATPase subunit 6 (A6) pre-mRNA, a specificity previously observed for KREN2-isolated editosomes (Fig. 4A). Only KREPB8 editosomes cleave the standard deletion site substrate based on A6 pre-mRNA, a specificity previously observed for KREN1-isolated editosomes (Fig. 4B). KREN3-specific cleavage was previously demonstrated in vitro using the COIIcisU1 substrate derived from cytochrome oxidase II (COII) pre-mRNA, which by chance also contains a distinct cleavage site specifically recognized by KREN2 (3). To simultaneously assess cleavage activity of all three editing endonucleases, COIIcisU1 was modified by adding an unpaired U in the predicted helix between the KREN2 and KREN3 cleavage sites to create a KREN1-specific cleavage site, thereby making the ‘triple site’ substrate (Fig. 4C). Cleavage specificities at each of the three sites are demonstrated by TAP-isolated KREN1, KREN2, and KREN3 editosomes (Fig. 4D). For each endonuclease-specific editosome, a single predominant cleavage product is produced. Cleavage by KREPB6, KREPB7, and KREPB8 editosomes mirrors the specificity of KREN3, KREN2, and KREN1 editosomes, respectively (Fig. 4E). Because KREPB8 editosomes had lower amounts of cleavage activity than KREN1 editosomes, assays in Fig. 4E were biased toward deletion cleavage activity by addition of 1 mM ADP, which is known to stimulate deletion and inhibit insertion cleavage (44).

**Analysis of KREPB6, KREPB7, and KREPB8 Editosomes in the Absence of Partner Endonuclease**—To examine how the editing endonucleases affect protein interactions within each type of editosome, TAP-tagged KREPB6, KREPB7, or KREPB8 were constitutively expressed in the background of the respective endonuclease conditional knock-out cell lines. In these BF cell lines, both endogenous endonuclease alleles have been eliminated by homologous recombination, and a tetracycline-inducible ectopic endonuclease allele introduced into the rDNA locus. Removal of tetracycline eliminates endonuclease expression, and causes growth defects after 3 days. Expression of the TAP-tagged protein was detected by recombinant peroxidase anti-peroxidase Western blot analysis (data not shown). Each

**TABLE 1**

Editosome proteins detected by mass spectrometry of TAP-tagged KREPB6 (B6 tag), KREPB7 (B7 tag), and KREPB8 (B8 tag) complexes that were isolated by sequential IgG and calmodulin affinity chromatography

| Proteins | GeneDB code | Function | B6 tag | B7 tag | B8 tag |
|----------|-------------|----------|--------|--------|--------|
| KREPA1   | Tb927.2.2470| Interaction | ✓      | ✓      | ✓      |
| KREPA2   | Tb927.10.8210| Interaction | ✓      | ✓      | ✓      |
| KREPA3   | Tb927.8.620 | Interaction | ✓      | ✓      | ✓      |
| KREPA4   | Tb927.10.5110| Interaction | ✓      | ✓      | ✓      |
| KREPA5   | Tb927.8.680 | Interaction†| ✓      | ✓      | ✓      |
| KREPA6   | Tb927.10.5120| Interaction | ✓      | ✓      | ✓      |
| KREN1    | Tb927.1.1690| Endonuclease ND | ND     | ND     | ND     |
| KREN2    | Tb927.10.5440| Endonuclease ND | ND     | ND     | ND     |
| KREN3    | Tb927.10.5320| Endonuclease ND | ND     | ND     | ND     |
| KREB4    | Tb11.02.0490| Interaction | ✓      | ✓      | ✓      |
| KREB5    | Tb11.03.0180| Interaction | ✓      | ✓      | ✓      |
| KREB6    | Tb927.3.3990| Interaction† | ND     | ND     | ND     |
| KREB7    | Tb927.160.4130| Interaction† | ND     | ND     | ND     |
| KREB8    | Tb927.8.5690| Interaction† | ND     | ND     | ND     |
| KREX1    | Tb7.1.1070 | Exonuclease ND | ND     | ND     | ND     |
| KREX2    | Tb10.20844 | Exonuclease ND | ND     | ND     | ND     |
| KREX3    | Tb927.160.2970| RNA ligase ND | ND     | ND     | ND     |
| KREX4    | Tb927.1.3030| RNA ligase ND | ND     | ND     | ND     |
| KREX5    | Tb927.7.1550| TUTase ND | ND     | ND     | ND     |

*Indicates protein identified, superscript 1 indicates that only a single tryptic peptide was detected. †Denotes putative function. ND, not detected by mass spectrometry.

**FIGURE 3.** Pre-cleaved editing assays of TAP-isolated complexes. 20 S glycerol gradient fraction from purified mitochondria is used as a control. A, pre-cleaved insertion assay demonstrates that complexes isolated via tagged KREPB6, KREPB7, and KREPB8 all have editing TUTase and ligase activities. Asterisks denote the location of radiolabel. B, pre-cleaved deletion assay demonstrates that these complexes also have editing exonuclease and ligase activities.
KREN3 complexes isolated from cells expressing KREN1 do not appear to be primarily intact editosomes, as only signals for KREPA2, KREL1, and KREPA6 are robust, whereas signals for KREPB1, KREPA3, KRET2, and KREL2 are weak or undetectable. By comparison, signals for KREPA2, KREL1, and KREPA6 appear to slightly increase in KREPB8 complexes isolated from KREN1-repressed cells, whereas the weak signals for KREPB1 and KREL2 are lost. Analysis of these complexes was further supplemented by tandem mass spectrometry to detect editosome proteins (supplemental Table S1), and combined with Western blot and adenylation data to generate an overview of editosome composition in the presence or absence of endonuclease expression (Fig. 5B). Direct interactions between some editosome proteins have previously been characterized, and are summarized in Fig. 5C (22). Together, these data indicate that the presence of endonuclease is required for stable association of the heterotrimeric insertion subcomplex (KRET2-KREPA1-KREL2) in editosomes isolated via KREPB6 and KREPB7. Analysis of KREPB8 editosomes is complicated by the fact that even in the presence of KREN1 expression, editosome subcomplexes predominate. In an effort to understand whether the C-terminal TAP tag on KREPB8 interferes with isolation of intact editosomes, an N-terminal TAP tag KREPB8 was constructed, and the expression of the two versions compared in PF 29.13 cells. Although the N-terminal TAP tag KREPB8 is not expressed as well as the C-terminal version, Western analysis of TEV eluates indicate that both versions isolate robust editsome signals for KREPB1, KREPA2, KREL1, and KREPA3 (supplemental Fig. S2). However, subsequent purification via calmodulin affinity reveals that whereas the N-terminal-tagged KREPB8 isolates only 20 S editsome, C-terminal-tagged KREPB8 isolates both 20 S editsome and a significant amount of subcomplexes at lower S values. Therefore, the differential results observed for KREPB8 appear to be related to the location of the tag. Unfortunately, the lower expression levels of the N-terminal TAP-tagged KREPB8 were insufficient for isolating editosomes from BF cells (data not shown).

Pre-cleaved insertion and deletion editing assays were used to determine whether complexes isolated via tagged KREPB6, KREPB7, or KREPB8 retained activities in the absence of the respective endonuclease. To increase the signal to noise ratio, editing activity was assessed using TEV eluates, which have greater amounts of complex. Similar results were obtained for KREPB6, KREPB7, and KREPB8 complexes: both insertion and deletion editing activities were observed in the presence of endonuclease expression, and when endonuclease was repressed only deletion editing activity was retained, and insertion editing activity was lost (Fig. 6). This result is consistent with the observation that the heterotrimeric insertion subcomplex, which contains TUTase KRET2, is lost in the absence of endonuclease.

**Analysis of Editsome Isolated from Cells Co-expressing Differentially Tagged Endonuclease**—To assess whether the editing endonucleases form homodimers in the context of the editsome, V5-epitope-tagged versions of KREN1, KREN2, and KREN3 were transfected into cells that already expressed TAP-
**Editosome RNase III Endonucleases Do Not Form Homodimers**

**A Pre-cleaved Insertion:**

| RNA | Insertion Product |
|-----|------------------|
| N3  | E                |
| N2  | R                |
| N1  | Edit inserted    |

**B Pre-cleaved Deletion:**

| RNA | Deletion Product |
|-----|------------------|
| N3  | E                |
| N2  | R                |
| N1  | UUU              |

Western blot signal for V5-tagged endonuclease was consistently more intense than that for TAP-tagged endonuclease in these experiments. Editosomes were isolated via TAP purification or immunoprecipitation from cell lysates of endonuclease co-expressing cell lines KREN1\textsubscript{TAP}, KREN2\textsubscript{V5}, KREN2\textsubscript{TAP}, KREN2\textsubscript{V5}, and KREN3\textsubscript{TAP}, KREN3\textsubscript{V5} as well as parental lines TAP-KREN1, TAP-KREN2, and TAP-KREN3. Parallel immunoprecipitations using lysate from equivalent cell numbers were performed using mouse monoclonal antibodies against KREPA2, V5, and HSP70 bound to goat anti-mouse-conjugated magnetic beads, as well as beads without antibody. Samples of purified editosomes were then resolved on 10% SDS-PAGE, transferred to nylon membrane, and simultaneously probed for editosome proteins using mouse monoclonal antibodies against KREPA1, KREPA2, KREL1, and KREPA3 as well as rabbit polyclonal antibody against V5-tagged endonuclease. Using quantitative infrared scanning for Western analysis, mouse primary antibodies were detected using IRDye800-conjugated goat anti-mouse secondary antibody, whereas anti-V5 rabbit antibody was detected using IRDye680-conjugated goat anti-rabbit secondary antibody. For each endonuclease, the absence of V5 signal in TAP-isolated editosomes indicates that the TAP-tagged and V5-tagged endonucleases do not co-exist in the same editosome (Fig. 7).

Immunoprecipitation using anti-V5 antibody effectively isolates editosomes only from cells that express V5-tagged endonuclease, indicating that the tagged protein incorporates into the editosome. Immunoprecipitation of editosomes via antibody against KREPA2 shows a significant signal for V5-tagged endonuclease, indicating that a large proportion of editosomes contain this tagged protein. Comparison of the relative amount of signal for editosome proteins KREPA1, KREPA2, KREL1, and KREPA3 between TAP-isolated and KREPA2-immunoprecipitated editosomes indicates that sufficient amounts of editosome are present in TAP-isolated editosomes to detect the V5 signal, if it was present. The lack of editosome signals in immunoprecipitations by
Fig. 7. Western analysis shows that a single copy of an endonuclease is present in an editosome. For each endonuclease (A, KREN1; B, KREN2; C, KREN3) editosomes were TAP purified (TAP) or immunoprecipitated (by antibodies against KREPA2 or V5 epitope) and analyzed by Western analysis. Blots were simultaneously probed with antibodies that recognized editosome proteins KREPA1, KREPA2, KREPA3, and KREL1 (top panel) as well as the V5 epitope (bottom panel). First 5 lanes represent samples purified from cells expressing both TAP-tagged and V5-tagged endonuclease (TAP+V5); sixth to eighth lanes are controls from cells expressing TAP-tagged endonuclease alone (TAP alone). Ninth and tenth lanes (Lysates) compare lysates from equivalent cell numbers used in the first 5 lanes or the sixth to eighth. White arrows in the first lane (TAP) highlight that TAP-isolated editosomes lack V5-tagged endonuclease despite co-expression of V5-tagged endonuclease. Black arrows highlight V5-tagged endonuclease detected in αKREPA2 (αA2 IP) samples; using the common set of editosome proteins (A1, A2, L1, and A3) as a reference, sufficient amounts of editosome are present in the first lane to detect V5-tagged endonuclease if it were present. Expression of the V5-tagged endonuclease is also shown by αV5 immunoprecipitation (αV5 IP), as editosome is only isolated from cells that express V5-tagged endonuclease (compare the αV5 immunoprecipitates in the fifth lane 5 to the seventh lane). Specificity of immunoprecipitation is shown by lack of editosome signal in lanes for beads without antibody (Beads–Ab) or with non-editosome antibody (αHSP70). Bands labeled by an asterisk represent nonspecific binding of antibody to the Protein A moiety on TAP-tagged endonuclease, and demonstrate a decreased amount of TAP-tagged endonuclease in cells that also express V5-tagged endonuclease compared with parental cells that express TAP-tagged endonuclease alone.

HSP70 or beads lacking secondary antibody demonstrate the specificity of the immunoprecipitations by anti-KREPA2 and anti-V5 antibodies.

Discussion

The data presented here show that protein pairs KREPB6/KREN3, KREPB7/KREN2, and KREPB8/KREN1 associate with the same common set of editosome proteins, with the exception of the preferential association of KREN1 with KREPB8/KREN1 editosomes. Although all three editosomes can perform pre-cleaved insertion and deletion editing, each editosome has a particular endonucleolytic cleavage specificity. Repression of partner endonuclease expression caused a preferential loss of components of the insertion subcomplex in TAP-purified KREPB6 or KREPB7 complexes, suggesting a more stable structural association between KREPB6 and KREPB7 with the deletion subcomplex (Fig. 5). Although experiments with KREPB8 complexes were complicated by the inability to isolate predominantly intact editosomes in the presence of KREN1 expression, the results do show preferential association of the deletion subcomplex with KREPB8. Reciprocally, these experiments indicate that the endonucleases are structurally associated with the insertion subcomplex (Fig. 5). Furthermore, TAP-isolated editosomes from cells that co-express the same endonuclease as TAP-tagged and V5-tagged versions lacked V5-tagged endonuclease, indicating that only one endonuclease was present in each editosome. These and previously published data suggest that KREPB6, KREPB7, and KREPB8 are important for endonuclease activity and specificity. They also indicate direct or indirect associations between the partner B proteins and the endonucleases and associations of the former with deletion subcomplexes and the later with insertion subcomplexes. The function of the KREPB6, KREPB7, and KREPB8 proteins and their associated complexes may be to accommodate recognition of numerous editing sites with different RNA sequences and structures.

The data presented here contribute insight into different editing endonuclease interactions within their respective editosomes, and support a novel mode of RNase III function. Isolation of distinct KREN1, KREN2, and KREN3 editosomes using tagged KREPB6, KREPB7, and KREPB6, respectively, provide an independent verification of the results previously obtained by directly tagging the endonucleases (3, 4). Mass spectrometry of these complexes indicates that the same proteins are purified using tagged KREN1 and KREPB8, results recapitulated with KREN2 and KREPB7 as well as KREN3 and KREPB6 (Table 1). SYPRO Ruby profiles of KREPB6, KREPB7, and KREPB8 editosomes look extremely similar, with some expected exceptions (Fig. 2A). Bands consistent with the sizes for KREN3, KREN2, and KREN1 were only found in KREPB6, KREPB7, and KREPB8, respectively. A unique band for KREN1 was not visible in KREPB8 editosomes analyzed on this gel because it overlaps with KREX2 at this resolution (data not shown). Although all three of these editosomes are competent for both pre-cleaved insertion and deletion editing (Fig. 3), KREN1-specific deletion site cleavage activity is only found with KREPB8 editosomes, KREN2-specific insertion site cleavage activity with KREPB7 editosomes, and KREN3-specific insertion site cleavage activity with KREPB6 editosomes (Fig. 4). The differential substrate specificity of each editing endonuclease has previously been examined using in vitro cleavage
assays with distinct RNA substrates, primarily using separate pre-mRNA and cognate gRNA. In this work we present a single cis-guided RNA substrate that contains distinct cleavage sites for KREN1, KREN2, and KREN3 (Fig. 4C). This triple site sub- strate, derived from the COII pre-mRNA sequence, provides a way to simultaneously assay cleavage activity of all three endo- nucleases in a single assay.

Further analyses indicate some unexpected differences be- tween TAP-isolated KREN1, KREN2, and KREN3 editosomes compared with KREPB8, KREPB7, and KREPB6 editosomes. Although previous experiments showed no discernable differ- ence in the relative levels of KREL1 or KREL2 adenylation signal for isolated KREN1, KREN2, and KREN3 editosomes in com- parison to ~20 S mitochondrial control (4), a significant reduc- tion of the KREL2 signal was apparent in KREPB6, KREPB7, and KREPB8 editosomes (Fig. 2C). The cause of decreased KREL2 association in these editosomes is unclear. Perhaps steric interference by the C-terminal TAP tag on KREPB6, KREPB7, and KREPB8 prevents robust association of KREL2. Comparison of editosomes isolated via N-terminal or C-termi- nal TAP-tagged KREPB8 (see below) indicate interference with the association of components of the heterotrimeric insertion subcomplex (KRET2-KREPA1-KREL2), which is consistent with this hypothesis. Subsequent analysis of TAP-tagged KREPB6, KREPB7, and KREPB8 editosomes showed that association of KREL2 in the editosome was dependent on the expression of the partner endonuclease. This suggests that the substoichiometric amount of KREL2 found in KREPB8, KREPB7, and KREPB6 editosomes may indicate that a subset of the isolated complexes lack endonuclease.

Another notable difference between editosomes isolated via tagged endonuclease compared with tagged partner protein is the detection of KREX1 in KREPB7 and KREPB6 editosomes (Fig. 2B). Analyses by SYPRO Ruby-stained gel and mass spec- trometry only identified KREX1 in KREPB8 editosomes, how- ever, Western analysis detected substoichiometric amounts of KREX1 in KREPB7 and KREPB6 editosomes. In contrast, West- ern analysis of KREN1, KREN2, and KREN3 editosomes showed that the KREX1 signal was restricted to KREN1 edito- somes (Fig. 2D). One explanation for these results is that KREX1 might interact weakly with KREPB6, KREPB7, and KREPB8 complexes in the absence of endonuclease, and whereas the presence of KREN1 strengthens this interaction, the presence of KREN2 or KREN3 disrupts it. In this scenario, the substoichiometric amounts of KREX1 in KREPB6 and KREPB7 samples reflect complexes that lack endonuclease, whereas the stoichiometric amounts of KREX1 in KREPB8 samples reflect the stabilizing presence of KREN1. Analysis of glycerol gradient fractions for KREPB6 seems to be consistent with this hypothesis, as the signal for KREX1 peaks in lower S value complexes ( supplemental Fig. S1). The peak signal for KREX1 in KREPB7 complexes overlaps with the ~20 S edito- some, but still appears at a lower S value than that observed with KREPB8 complexes. Whether KREX1 and KREN2 coexist within particular ~20 S KREPB7 complexes or whether KREX1 resides in a subpopulation of ~20 S complexes lacking KREN2 is unknown. The identification of substoichiometric amounts of KREX1 in KREPB7 and KREPB6 complexes may reflect tran- sient in vivo dynamics of protein association, particularly if endonucleases are exchanged during the editing process.

Examination of tagged KREPB6, KREPB7, and KREPB8 complexes in the presence or absence of endonuclease expression provides critical insight into the role of the endonucleases in editosome architecture. In the absence of endonuclease expres- sion, KREPB6, KREPB7, and KREPB8 associate with an edito- some subcomplex that notably lacks the heterotrimeric inser- tion subcomplex (Fig. 5). KREPB6, KREPB7, and KREPB8 therefore do not require their partner endonuclease to bind to other editosome proteins and the stable association of the KRET2-KREPA1-KREL2 subcomplex within the rest of the edi- tosome involves endonuclease. The partial reduction of KREPA3 and KREPA6 in KREPB6 and KREPB7 subcomplexes isolated in the absence of endonuclease is consistent with the previous characterization of these proteins as a “bridge” between the deletion and insertion heterotrimeric subcomplexes, as the substoichiometric amount of these proteins may reflect the destabilizing effect caused by the absence of the insertion subcomplex. KREPA3 might directly interact with the endonucleases, as its presence has been shown to be crucial for cleavage activity (45). One striking difference between KREPB6, KREPB7, and KREPB8 complexes is the extent of reduction of KREPA6 in the absence of endonuclease expres- sion (Fig. 5A). Although KREPB6 editosomes lose virtually all KREPA6 signal, KREPB7 editosomes have a modest reduction of KREPA6, and KREPB8 editosomes appear to have slightly more KREPA6 when endonuclease is not expressed. Although the precise stoichiometry of editosome proteins is not clear, purified recombinant KREPA6 has been shown to form dimers and tetramers in vitro, suggesting multiple copies of KREPA6 are present in editosomes in vivo (22, 46). The differential amounts of KREPA6 between KREPB6, KREPB7, and KREPB8 complexes reflect differences in the architecture of KREN3, KREN2, and KREN1 editosomes. These differences may be a result of direct interactions between KREPA6 and each endonuclease, or indirect via proteins that require the endonuclease to associate with the editosome. Analysis of KREPB8 complexes is somewhat hampered by the inability to isolate complete edi- tosomes in the presence of KREN1 expression. Comparison of N-terminal and C-terminal TAP-tagged versions of KREPB8 expressed in PF cells indicates that the TEV eluates of both versions contain intact ~20 S editosomes, with a much greater yield observed with C-terminal tagged KREPB8 ( supplemental Fig. S2). However, elution from calmodulin shows that whereas the N-terminal KREPB8 retains a fairly uniform ~20 S edito- some composition, the C-terminal KREPB8 has a predominant amount of subcomplex that lacks KREPA1. The same subcom- plex is evident in C-terminal KREPB8 complexes isolated from the BF KREN1 conditional knock-out background, and it apparently predominates over intact ~20 S editosomes. Unfortunately, the lower expression levels of the N-terminal TAP- tagged KREPB8 in BF cells prevented isolation of sufficient edito- some for analysis (data not shown). Despite the technical issues with KREPB8 TAP tag isolation from BF cells, the results obtained are consistent with those for KREPB6 and KREPB7, in that these proteins associate with the part of the editosome that contains the deletion subcomplex.

**Editosome RNase III Endonucleases Do Not Form Homodimers**
The experiments presented here also provide the first mass spectrometry data of purified editosomes from the bloodstream from trypanosomes. All of the proteins previously identified in PF TAP-tagged editosomes were also identified in BF, with the exception of KREN1. The failure to detect KREN1 is likely due to the technical difficulties associated with the C-terminal tag of KREPB8 discussed above, as KREN1 activity has been shown in BF extracts (25). No novel editosome proteins were identified with high confidence in BF samples. Therefore, the significant differences between RNA editing in PF and BF (47) do not appear to result from substantial compositional differences in ~20 S editosomes.

A central question in characterizing editing endonuclease function has been the nature of the RNase III catalytic site. It seems likely that editing endonucleases have a dimeric catalytic site composed of two opposing RNase III domains like all characterized RNase III endonucleases. The conventional possibility that the editing endonucleases form homodimers has been implied by circumstantial evidence in previous experiments. A ~100-kDa protein that cross-linked in vitro with TAP-tagged KREN1 but not KREN2 editosomes was suggested to be endogenous KREN1, implying the possibility that it exists as a homodimer in vivo (48). An alternate possibility is that this protein corresponds to KREX1, which is also unique to KREN1 editosomes. In another experiment, the recombinant KREN1 protein was reported to cleave a model editing substrate in vitro (49). This activity reflects a function outside the context of the editosome, perhaps involving the formation of homodimers in vitro. Nevertheless, whereas most RNase IIIIs cleave both strands of double-stranded RNA, only the pre-mRNA strand is apparently cleaved during editing. Mutational analyses have shown that the RNase III motifs of KREN1, KREN2, and KREN3 are essential for cleavage activity (3, 24, 25), in contrast, the degenerate RNase III motifs of KREPB4 and KREPB5 are not essential for cleavage activity, suggesting that they are catalytically inactive.3 Based on our data and these considerations we hypothesize that KREPB4 and/or KREPB5 form intermolecular heterodimers with KREN1, KREN2, and KREN3, so that only pre-mRNA is cleaved during editing.

The experiments presented herein suggest that the editing endonucleases do not form homodimers within the editosome because V5- and TAP-tagged endonucleases did not co-purify from cells in which both are expressed (Fig. 7, white arrows). The lack of detection of V5-tagged endonuclease is not due to limited sensitivity because endonucleases that are immunoprecipitated with monoclonal antibody specific to KREPA2 (present in all editosomes) had robust Western signal for V5-tagged endonuclease (Fig. 7, black arrows). Lysates from cells co-expressing both tagged endonucleases have 5.3–12.4-fold less Western signal for TAP-tagged endonuclease compared with parental cells expressing only TAP tag endonuclease, suggesting that the robust Western signal for V5-tagged endonuclease reflects the presence of more V5-tagged versus TAP-tagged endonuclease per cell. The apparent predominance of the smaller V5-tagged endonuclease may reflect greater stability and/or preferential incorporation into editosomes compared with TAP-tagged endonuclease. It is possible that the lack of V5-tagged endonuclease in TAP-tagged purified editosomes is because two C-terminal-tagged endonucleases might not be able to be incorporated into editosomes, however, this is unlikely because cells that exclusively express TAP-tagged endonucleases (which are essential for survival) grow normally (3). The reciprocal experiment of assaying for TAP-tagged endonuclease in anti-V5 immunoprecipitated editosomes was not feasible, as the Protein A moiety of the TAP tag binds to the Fc portion of the immunoprecipitating antibody. Overall the apparent lack of editosomes containing both types of tagged endonucleases indicates that each editosome contains only one copy of each editing endonuclease.

How three distinct editosomes function in concert in vivo is unknown, but the results we show here offer some tantalizing clues. The fact that the ~20 S editosomes require endonucleases for stable association of the insertion subcomplex reveals an important structural role for these catalysts. This may imply that editing of the different insertion and deletion editing sites may entail dynamic exchange of editosome proteins and/or subcomplexes as the editosome encounters the different sites. By this model the editosome composition would be adapted to the characteristics of the editing site. Alternatively, editosome composition could be relatively stable and different editosomes would be recruited as specified by the characteristics of the editing site. Both models are consistent with in vivo editing, which does not appear to require precise 3′ to 5′ progression in the order in which the sites are edited (50). How the editing endonucleases recognize their distinct editing sites and the roles that KREPB4 and KREPB5 play in vivo require additional exploration.

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