ENDONUCLEASE ASSOCIATIONS WITH THREE DISTINCT EDITOSOMES IN TRYPANOSOMA BRUCEI

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Running Head: Editosome RNase III endonucleases do not form homodimers

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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 of 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 was 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 while the KREN1, KREN2 and KREN3 endonucleases associate with insertion subcomplex.

Transcripts from most mitochondrial genes in Trypanosoma brucei require post-transcriptional RNA editing, in which uridine nucleotides (Us) are inserted or deleted in order 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 ~20S 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 (Figure 1A). While these ~20S 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 (5;6), MRB1 (7-10), RBP16 (11;12), REAP1 (13), TbRGG1 (14), TbRGG2 (15), KRET1 (16;17), MEAT1 (18), KPAP1 (19)] have functions which 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 reconstitution 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 endonuclease 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

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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 which is required for cleavage activity (26). All characterized RNase III endonucleases function as dimers: intermolecular homodimers such as archetypal E. 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 dsRNA 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). While 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 (Schnaufer, et al. unpublished). 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 archetypal 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 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. brucei* strain 427 genomic DNA and cloned into the pLEW-MHTAP plasmid (33). Oligos for PCR amplification were
ATAAAGCTTATGACTCCTGTATTTGGTAC (5571) and AAAGGATCCCCTCATCAAGACAAATCCTTT C (5572) for KREPB6; ATAAAGCTTATGTTTCAAAAAGCACTTCGT C (5573) and CGAGGATCCCACACCTTTAGAGAGTGATT GAG (5574) for KREPB7; ATAAAGCTTATGCACCGCGGACCGCCGG (5602) and AAAGGATCCCAGCAAATTCATTGCTGCAA G (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 phleomycin 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 HindIII and BamHI and cloned into the same sites in pHD1344tub (3) to make pHD1344tub-KREPB6-TAP, pHD1344tub-KREPB7-TAP, and pHD1344tub-KREPB8-TAP. NotI digested plasmids were then transfected into their respective BF endonuclease conditional knockout cells lines: KREPB6 into RKO-KREN3-KREPB6-TAP, RKO-KREN2-KREPB7-TAP, and KREPB8 into RKO-KREN1. Cell lines RKO-KREN3-KREPB6-TAP, RKO-KREN2-KREPB7-TAP, and RKO-KREN1-KREPB8-TAP 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 and 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 that endonuclease (4). Cell lines KREN1TAP-KREN1V5, KREN2TAP-KREN2V5, and KREN3TAP-KREN3V5 were isolated by concurrent phleomycin and 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 to 72 hours with 200 to 500 ng/mL tetracycline, and 1-2 L of cells were harvested at ~2x10^7 cells/mL. Crude mitochondrial preparations were made from harvested cells, lysed in 20 mL IPP150, 1% Triton X-100, Complete protease inhibitors at 4°C, and subsequently clarified by centrifugation at 10,000 x 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 hour) fractionation at 38,000 rpm at 4°C in SW-40 rotor (Beckman). For BF cell lines RKO-KREN3-KREPB6-TAP and RKO-KREN2-KREPB7-TAP, and RKO-KREN1-KREPB8-TAP, expression of the TAP-tagged protein is constitutive, while expression of the endonuclease regulated by tetracycline. To modulate endonuclease expression, cells were centrifuged at 1300 x g for 10 minutes, resuspended in media lacking tetracycline, then recentrifuged and resuspended again. Cells were then split into media with (expressed) or without (repressed) tetracycline. After 72 hours of growth, cells at ~2e6 cells/mL were harvested for all cell lines, 2 L each with and without tet for RKO-KREN3-KREPB6-TAP and RKO-KREN2-KREPB7-TAP; 6 L each with and without tet for RKO-KREN1-KREPB8-TAP. To harvest, cells were centrifuged at 1300 x g for 10 minutes, resuspended in 30 mL media, recentrifuged, and cell pellets flash frozen on liquid nitrogen. Frozen cells were resuspended in 20 mL IPP150, 1% Triton X-100, Complete protease inhibitors (Roche) 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 manufacturer’s protocol (Molecular Probes), and bands visualized using Alpha Innotech Alphalager EP. For Western analyses in Figures 2 and 7 as well as Supplemental Figure 1, 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 Figure 2 or 1:10,000 rabbit polyclonal antibody against the V5 epitope (GenScript) for Figure 7 in Odyssey blocking buffer. After 4 washes with 1X PBS-T, blots were probed with 1:15,000 IRDye680 conjugated goat anti-rabbit (LiCor) and IRDye800 conjugated goat anti-mouse (Rockland) secondary antibodies. Blots were washed 4 times with 1X PBS-T and 3 times with 1X PBS, dried, and scanned with a LiCor Odyssey scanner, and analyzed with Odyssey V3.0 software. For western analyses in Figure 5 and Supplemental Figure 2, blots were developed with ECL kit (Pierce) per manufacturer’s instructions.

Mass spectrometry. Proteins in editosome samples isolated by tagged KREPB6, KREPB7, and KREPB8 were denatured with 8M 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 cataloged in Supplemental Table 2.

Adenylation assays. Auto-adenylation KREL1 and KREL2 with [α-32P]-ATP was performed as previously described (38). Proteins were resolved on 10% Criterion (BioRad) SDS-PAGE gels that were then fixed in 50% methanol/10% acetic acid, equilibrated in 10% methanol/4% glycerol, dried, and analyzed by PhosphorImager (Molecular Dynamics).

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 cleavage product as previously described (3) using radiolabeled A6-eES1 pre-mRNA with gA6[14] gRNA and radiolabeled A6short/TAG.1 pre-mRNA with D34 gRNA, respectively. Triple-site substrate (GGGCAUUAAUAGAUUAACCCUGGUAG UGUAUAAAGGAGGGAAAGGUAUA AUCUAUAUGAAAGGGGAUUUUAAGAC) was derived from COIIcisU1, and assayed as previously described for that substrate for Figure 4D (3). For Figure 4E, 1 mM ADP was added to all assays to stimulate deletion cleavage activity. Each editing assay used 15 µL of sample.

Complex purification from cell lines co-expressing tagged endonucleases. Cell lines (KREN1TAP-KREN1V5, KREN2TAP-KREN2V5, KREN3TAP-KREN3V5, TAP-KREN1, TAP-KREN2, and TAP-KREN3) were grown in the presence of 200 ng/mL tetracycline for 48 to 72 hours, and harvested at ~2x10^7 cells/mL. Aliquots of ~2x10^9 total cells were set aside for immunoprecipitations. For cell lines expressing V5-tagged endonuclease, TAP-tagged complexes were isolated from the remaining (~4x10^10) cells as described above. For samples from parental TAP-KREN1, TAP-KREN2, and TAP-KREN3 cell lines, calmodulin eluate fractions 2-4 were pooled and 70 µL combined with 30 µL 2x SDS sample buffer, and 20-25 µL used for western. For KREN1TAP-KREN1V5, KREN2TAP-KREN2V5, and KREN3TAP-KREN3V5 cell lines, calmodulin eluate fractions 2-4 were pooled and 680 µL incubated with 30 µL StrataClean Resin (Stratagene) for 10 minutes on ice to concentrate samples. Resin was spun for 2 minutes at 2,000 x g, supernatant was discarded, complexes resuspended in 65 µL 2x 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 1X PBS/0.1% BSA and incubated with 0.5 µg Protein A (Sigma) to minimize non-specific binding of the TAP-tagged endonucleases to the Fc portion of bound IgG. Beads were then incubated with 500 µL 1X PBS/1% BSA and either 500 µL mouse antibody specific for KREPA2 (36), 500 µL mouse antibody (mAb 78) specific for heat shock protein 70 (43), or the 0.5 µL mouse antibody specific for V5 epitope tag (Invitrogen) for two hours rotating at 4°C. Beads were then washed four times with 1
mL cold IPP150 (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40) and incubated for two hours rotating at 4°C with cleared lysate (5x10⁸ cells lysed in IPP150 with 1% Triton X-100, followed by centrifugation at 10,000 x g at 4°C). After incubation, supernatant was removed, and beads were washed four times with 1 mL IPP150. Complexes bound to beads were eluted with 100 μL of 2x SDS sample buffer, heated for 5 minutes at 95°C, and 12 μL used for western. Sample volumes (as stated above) were adjusted to yield similar intensities of KREPA1, KREPA2, KREL1, and KREPA3 signal.

RESULTS

Analysis of TAP-tag isolated KREPB6, KREPB7 and KREPB8 complexes. Tandem Affinity Purification (TAP) tags (4;35) were added to the C-terminus of KREPB6, KREPB7, or KREPB8, and subsequently expressed in PF 29.13 cells (Figure 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 was 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 rPAP, 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 (Figure 2A). While 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 a few distinct proteins.

TAP-isolated complexes were further analyzed by Western blot and adenylation assays. A 20S 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 lower amount of KREPA1 and KREPA3 signal relative to KREPA2 and KREL1 signal (Figure 2B, top). Simultaneous probing for KREX1 reveals robust signals for KREPB8 and 20S mitochondrial control and much weaker signals for KREPB7 and KREPB6 (Figure 2B, middle). Quantitation of the KREX1 signal intensity normalized to 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 to KREPB8, while 20S control has 1.3 fold less. In contrast, among editosomes isolated via TAP-tagged endonuclease, only KREN1 has detectable KREX1 signal (Figure 2D). Western analysis of KREPB6, KREPB7 and KREPB8 complexes using antibody against the calmodulin binding peptide (αCBP) detects the tagged protein, and shows single bands for KREPB6 and KREPB7 that have similar apparent sizes, while multiple bands for KREPB8 suggest a predominant intact protein with minor degradation products below (Figure 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 20S mitochondrial control (Figure 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. While each complex contains the 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 reveals 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 (Figure 3). KREPB6, KREPB7 and KREPB8 editosomes were all able to specifically add Us and ligate RNAs (Figure 3A) as well as specifically remove Us and ligate RNA (Figure 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 (Figure 4A). Only KREPB8 editosomes cleave standard deletion-site substrate based on A6 pre-mRNA, a specificity previously observed for KREN1-isolated editosomes (Figure 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 (Figure 4C). Cleavage specificities at each of the three sites are demonstrated by TAP-isolated KREN1, KREN2, and KREN3 editosomes (Figure 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 (Figure 4E). Because KREPB8 editosomes had lower amounts of cleavage activity than KREN1 editosomes, assays in Figure 4E were biased towards 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 was constitutively expressed in the background of the respective endonuclease conditional knockout 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 rPAP western blot (data not shown). Each cell line was grown for 3 days with endonuclease either expressed or repressed, and cell lysates were used for TAP purifications. The composition of each TAP-isolated complex, with or without endonuclease expression, was analyzed by Western, adenylation, tandem mass spectrometry, and pre-cleaved editing assays. KREPB6 complexes isolated from cells expressing KREN3 have signals for KREPA1, KREPA2, KREL1, KREPA3, KRET2, and KREPA6 by Western blot, as well as KREL2 by adenylation (Figure 5A). With KREN3 repressed, however, only KREPA2 and KREL1 have robust signals, while KREPA1, KREPA3, KRET2, KREPA6, and KREL2 are lost or significantly diminished. Although KREPB7 complexes from KREN2-repressed cells appear to have reduced KREPA6, the extent of this reduction is not nearly as dramatic as with KREPB6 complexes from KREN3-repressed cells. Unlike KREPB6 and KREPB7, KREPB8 complexes isolated from cells expressing KREN1
do not appear to be primarily intact editosomes, as only signals for KREPA2, KREL1, and KREPA6 are robust, while signals for KREPA1, 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, while the weak signals for KREPA1 and KREL2 are lost. Analysis of these complexes was further supplemented by tandem mass-spectrometry to detect editosome proteins (Supplementary Table 1), and combined with Western and adenylation data to generate an overview of editosome composition in the presence or absence of endonuclease expression (Figure 5B). Direct interactions between some editosome proteins have previously been characterized, and are summarized in Figure 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 editosome signals for KREPA1, KREPA2, KREL1, and KREPA3 (Supplementary Figure 2). However, subsequent purification via calmodulin affinity reveals that while the N-terminally tagged KREPB8 isolates only 20S editosome, C-terminally tagged KREPB8 isolates both 20S editosome 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 if complexes isolated via tagged KREPB6, KREPB7, or KREPB8 retained activities in the absence of respective endonuclease. To increase 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 (Figure 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 editosomes isolated from cells co-expressing differentially tagged endonuclease. To assess whether the editing endonucleases form homodimers in the context of the editosome, V5-epitope tagged versions of KREN1, KREN2, and KREN3 were transfected into cells that already expressed TAP-tagged versions of the same endonuclease. If the editing endonucleases do form homodimers, then the V5-tagged endonuclease would be expected to co-purify with TAP isolated editosomes. Although expression of both TAP-tagged and V5-tagged alleles was detected by western blot for each cell line, the amount of TAP-tagged endonuclease was significantly reduced after introduction of V5-tagged endonuclease in comparison to parental cell lines that expressed TAP-tagged endonuclease alone (data not shown; Figure 7, see band marked by asterisk). Comparison of signal corresponding to TAP-tagged endonuclease in lysates from equivalent cell numbers decreased 8.6 fold (KREN1), 12.4 fold (KREN2), or 5.3 fold (KREN3) in cells expressing both tagged alleles versus the parental cell line just expressing TAP-tagged allele. 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_TAP-KREN1V5, KREN2_TAP-KREN2V5, and KREN3_TAP-KREN3V5 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, while 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 (Figure 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 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 V5 signal, if it were present. The lack of editosome signals in immunoprecipitations by HSP70 or beads lacking secondary antibody demonstrates 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 KREX1 with KREPB8/KREN1 editoromes. While all three editoromes can perform pre-cleaved insertion and deletion editing, each editorome 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 editoromes, suggesting a more stable structural association between KREPB6 and KREPB7 with the deletion subcomplex (Figure 5). While experiments with KREPB8 editoromes were complicated by the inability to isolate predominantly intact editoromes 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 (Figure 5). Furthermore, TAP-isolated editoromes 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 editoromes, and support a novel mode of RNase III function. Isolation of distinct KREN1, KREN2, and KREN3 editoromes using tagged KREPB8, 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 editoromes look extremely similar, with some expected exceptions (Figure 2A). Bands consistent with the sizes for KREN3, KREN2, and KREX1 were only found in KREPB6, KREPB7, and KREPB8, respectively. A unique band for KREN1 was not visible in KREPB8 editoromes analyzed on this gel because...
it overlaps with KREX2 at this resolution (data not shown). While all three of these editosomes are competent for both pre-cleaved insertion and deletion editing (Figure 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 (Figure 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 (Figure 4C). This “Triple-site” substrate, derived from COII pre-mRNA sequence, provides a way to simultaneously assay cleavage activity of all three endonucleases in a single assay.

Further analyses indicate some unexpected differences between TAP-isolated KREN1, KREN2, and KREN3 editosomes compared to KREPB8, KREPB7, and KREPB6 editosomes. While previous experiments showed no discernable difference in the relative levels of KREL1 or KREL2 adenylation signal for isolated KREN1, KREN2, and KREN3 editosomes in comparison to ~20S mitochondrial control (4), a significant reduction of KREL2 signal was apparent in KREPB6, KREPB7, and KREPB8 editosomes (Figure 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-terminal 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 a subset of the isolated complexes lack endonuclease.

Another notable difference between editosomes isolated via tagged endonuclease compared to tagged partner protein is the detection of KREX1 in KREPB7 and KREPB6 editosomes (Figure 2B). Analyses by SYPRO-Ruby stained gel and mass spectrometry only identified KREX1 in KREPB8 editosomes; however, Western analysis detected substoichiometric amounts of KREX1 in KREPB7 and KREPB6 editosomes. In contrast, Western analysis of KREN1, KREN2, and KREN3 editosomes showed that KREX1 signal was restricted to KREN1 editosomes (Figure 2D). One explanation for these results is that KREX1 might interact weakly with KREPB6, KREPB7, and KREPB8 complexes in the absence of endonuclease, and while 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, while the stoichiometric amounts of KREX1 in KREPB8 sample 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 Figure 1). The peak signal for KREX1 in KREPB7 complexes overlaps with ~20S editosome, but still appears at a lower S value than that observed with KREPB8 complexes. Whether KREX1 and KREN2 coexist within particular ~20S KREPB7 complexes or whether KREX1 resides in a subpopulation of ~20S complexes lacking KREN2 is unknown. The identification of substoichiometric amounts of KREX1 in KREPB7 and KREPB6 complexes may reflect transient 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 expression, KREPB6, KREPB7, and KREPB8 associate with an editosome subcomplex that notably lacks the heterotrimeric insertion subcomplex (Figure 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 editosome 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 expression (Figure 5A). While 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 editosomes 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 ~20S editosomes, with a much greater yield observed with C-terminal tagged KREPB8 (Supplemental Figure 2). However, elution from calmodulin shows that while the N-terminal KREPB8 retains a fairly uniform ~20S editosome composition, the C-terminal KREPB8 has a predominant amount a subcomplex that lacks KREPA1. The same subcomplex is evident in C-terminal KREPB8 complexes isolated from BF KREN1 conditional knockout background, and it apparently predominates over intact ~20S editosomes. Unfortunately, the lower expression levels of the N-terminal TAP-tagged KREPB8 in BF cells prevented isolation of sufficient editosome 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.

The experiments presented here also provide the first mass spectrometry data of purified editosomes from bloodstream form trypanosomes. All of the proteins previously identified in PF TAP-isolated 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 ~20S 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, recombinant KREN1 protein was reported to cleave a model editing substrate 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, 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, while most RNase IIIIs cleave both strands of dsRNA, 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
(Schnaufer et al., unpublished). 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 since V5- and
TAP- tagged endonucleases did not co-purify from
cells in which both are expressed (Figure 7, white
arrows). The lack of detection of V5-tagged
endonuclease is not due to limited sensitivity since
editosomes that are immunoprecipitated with
monoclonal antibody specific to KREPA2 (present
in all editosomes) had robust Western signal for
V5-tagged endonuclease (Figure 7, black arrows).
Lysates from cells co-expressing both tagged
endonucleases have 5.3 to 12.4 fold less Western
signal for TAP-tagged endonuclease compared to
parental cells expressing only TAP-tag
endonuclease, suggesting that the robust western
signal for V5-tagged endonuclease reflects the
presence of more V5-tagged vs. 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 to 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 since 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
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 ~20S 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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105
RNA 12, 1038-1049
711

FOOTNOTES

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The abbreviations used are: PF, procyclic form; BF, bloodstream form; tet, tetracycline; U, uridine nucleotide; gRNA, guide RNA; TAP, tandem affinity purification; CBP, calmodulin binding peptide.

FIGURE LEGENDS

Figure 1. A. Schematic representation of three distinct editosomes, each typified by a unique endonuclease. Box highlights the common set of 12 editosome proteins, with dotted outlines around heterotrimeric deletion (KREX2, KREPA2, KREL1) and insertion (KRET2, KREPA1, KREL2) subcomplexes. Shorthand for editosome protein names is outlined in Table 1. B. Diagram of TAP-tagged KREPB6, KREPB7 and KREPB8 proteins highlighting location of conserved C2H2 U1-like zinc finger (U1 ZnF), as well as TAP-tag domains calmodulin binding peptide (CBP), TEV protease cleavage site (indicated by caret), and Protein A. C. MUSCLE alignment (51) highlighting C2H2 ZnF (in gray) for KREPB6, KREP7, KREP8, KREN1, KREN2, and KREN3; dots represent gaps.

Figure 2. Analyses of complexes isolated via TAP-tagged KREPB6, KREPB7, and KREPB8. A. TAP-isolated complexes resolved by 10% SDS-PAGE and stained with SYPRO-Ruby. Predicted location for tagged protein indicated by asterisk. Bands predicted to be KREN3, KREN2, and KREX1 are highlighted. B. Western blot probed with antibodies recognizing editosome proteins KREPA1, KREPA2, KREL1, and KREPA3 (top panel) or KREX1 (middle panel). A second blot shows TAP-tagged protein via calmodulin binding peptide reactivity (CBP; bottom panel). ~20S glycerol gradient fraction from purified mitochondria is used as a control. C. Adenylation of TAP-isolated complexes indicates that the proportion of KREL2 signal is decreased relative to KREL1 in comparison to ~20S control. D. Western blot of editosomes isolated via TAP-tagged KREN1, KREN2, and KREN3 using antibodies recognizing editosome proteins KREPA1, KREPA2, KREL1, and KREPA3 (top panel) or KREX1 (bottom panel).
Figure 3. Pre-cleaved editing assays of TAP-isolated complexes. ~20S 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 in schematic denote location of radiolabel. B. Pre-cleaved deletion assay demonstrates that these complexes also have editing exoUase and ligase activities.

Figure 4. Editing endonuclease cleavage assays of TAP-isolated complexes. Reference ladders were generated by cleavage using alkaline hydrolysis (OH) or RNase T1 (T1). Arrows denote cleavage products. Asterisks in schematics denote location of radiolabel. A. KREB7 editosomes cleave standard insertion substrate A6-eES1 with gA6[14] gRNA, while KREPB6 and KREPB8 do not. Cleavage product in control reactions using ~20S glycerol gradient fraction from purified mitochondria is visible in the presence of gRNA (+g) but not in the absence of gRNA (-g). B. KREB8 editosomes cleave standard deletion substrate A6short/TAG.1 with D34 gRNA, while KREPB6 and KREPB7 do not. C. Sequence of “Triple-site” substrate RNA, which has distinct cleavage sites for KREN1, KREN2, and KREN3 endonucleases. Wedges indicate the cleavage site for each endonuclease. D. Cleavage of “Triple-site” substrate RNA by either ~20S glycerol gradient fraction from purified mitochondria, or TAP-isolated complexes via tagged KREN1, KREN2, or KREN3 endonuclease. Different sites cleaved by each endonuclease are indicated by arrows. E. Similar “Triple-site” cleavage assay using TAP-isolated complexes via tagged KREPB6, KREPB7, or KREPB8. Cleavage specificity of KREPB6, KREPB7, and KREPB8 complexes mirrors that observed for KREN3, KREN2, and KREN1 complexes, respectively.

Figure 5. Analyses of KREPB6, KREPB7, and KREPB8 editosomes in the presence or absence of respective endonuclease. A. TAP-tagged KREPB6 (B6TAP), KREPB7 (B7TAP), or KREPB8 (B8TAP) were expressed in the background of KREN3, KREN2, or KREN1 conditional knockout cell lines, respectively, and used to purify complexes from cells in which the cognate endonuclease was either expressed (Exp.) or repressed (Rep.). The presence of editosome components in isolated complexes was assessed by western blot (KREPA1, KREPA2, KREL1, KREPA3, KRET2, or KREPA6) or adenylation (KREL2). B. Schematic representation of isolated editosomes based on combined western, adenylation, and mass spectrometry (see Supplemental Tables 1 and 2) data to illustrate the effect of endonuclease repression on the composition of TAP-tagged KREPB6, KREPB7, and KREPB8 complexes. Decreased font size indicates that the relative amount of that protein appeared reduced by western analysis after endonuclease repression. An asterisk denotes that mass spectrometry did not detect this protein, but no corroborative evidence supports its absence. Note that in all cases, complexes isolated in the absence of partner endonuclease lack the insertion heterotrimeric subcomplex. C. Schematic of interactions and associations between editosome proteins. Previously characterized interactions between the common set of editosome proteins (22) are represented by solid black lines. Dashed black lines outline heterotrimeric insertion and deletion subcomplexes. Curved gray lines represent associations with insertion or deletion subcomplex for endonucleases or partner proteins, respectively. Dashed gray lines represent hypothetical interaction between endonucleases and KREPB4 or KREPB5 based on results shown in Figure 7.

Figure 6. TUTase activity is lost, but other editing activities remain in KREPB6, KREPB7 and KREPB8 complexes after endonuclease repression. Complexes purified by IgG affinity (TEV eluates) via tagged KREPB6 (B6TAP), KREPB7 (B7TAP), or KREPB8 (B8TAP) with partner endonuclease expressed (E) or repressed (R). Asterisks in schematics denote location of radiolabel. A. Pre-cleaved insertion assays show loss of TUTase activity and retention of ligase activity after endonuclease repression, consistent with the loss of KRET2 and retention of KREL1 shown in Figure 5A. B. Pre-cleaved deletion assays show both exoUase and ligase activities are retained after endonuclease repression.

Figure 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. Blots were
simultaneously probed with antibodies that recognize editosome proteins KREPA1, KREPA2, KREPA3, and KREL1 (top panel) as well as the V5 epitope (bottom panel). Lanes 1-5 represent samples purified from cells expressing both TAP-tagged and V5-tagged endonuclease (TAP+V5); lanes 6-8 are controls from cells expressing TAP-tagged endonuclease alone (TAP alone). Lanes 9-10 (Lysates) compare lysates from equivalent cell numbers used in lanes 1-5 or 6-8. White arrows in Lane 1 (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 Lane 1 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 αV5 IPs in Lane 5 to Lane 7). 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 asterisk represent non-specific binding of antibody to Protein A moiety on TAP-tagged endonuclease, and demonstrate decreased amount of TAP-tagged endonuclease in cells that also express V5-tagged endonuclease compared to parental cells that express TAP-tagged endonuclease alone.

**TABLES**

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. √ indicates protein identified, superscript 1 indicates that only a single tryptic peptide was detected, and ND indicates not detected by mass spectrometry. Asterisk denotes putative function. Shorthand names for editosome proteins indicated by underlined part of each full name.

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Figure 1

A

B6 N3
B7 N2
B8 N1 X1

X2 B5 B4 T2
X2 B5 B4 T2
X2 B5 B4 T2
A2 A6 A3 A1
A2 A6 A3 A1
A2 A6 A3 A1
L1 A4 A5 L2
L1 A4 A5 L2
L1 A4 A5 L2

B

KREP6
KREP7
KREP8

U1 ZnF

CBP- Protein A

CBP- Protein A

CBP- Protein A

C

KREP6
TGW. NRTENW. RSLLEPL. NSWSEIKG KRONIC LEM
KREP7
KRNVPFRPPEW EDLKEPV. LDMNSBG RENHAL MEM
KREP8
KLWLDLCTGW HELQEMGATMEVHEIC DRSEPHT LQY
KREP3
NLITDAAKNY EKLGKEEVYTPAKK HSTFGSNTMHTCERYLDS LAL
KREP2
LQFVQIFRIFQDGREGKLENSY MQKSS E VTPFQGREAAL LEM
KREP3
TAFYNAQPCR... STIZDDRLETSFSACG... TVGQVARVGLERMIE
Figure 2
Figure 3

A Precleaved Insertion:

B Precleaved Deletion:
Figure 5
Figure 6

A Precleaved Insertion:

B Precleaved Deletion:
Figure 7

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<th>TAP alone</th>
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A1

B

C

N1

N2

N3

N1V5

N2V5

N3V5
Endonuclease associations with three distinct editosomes in Trypanosoma brucei
Jason Carnes, Carmen Zelaya Soares, Carey Wickham and Kenneth Stuart

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