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# Editosome Accessory Factors KREPB9 and KREPB10 in *Trypanosoma brucei*

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Multiprotein complexes, called editosomes, catalyze the uridine insertion and deletion RNA editing that forms translatable mitochondrial mRNAs in kinetoplastid parasites. We have identified here two new U1-like zinc finger proteins that associate with editosomes and have shown that they are related to KREPB6, KREPB7, and KREPB8, and thus we have named them Kinetoplastid RNA Editing Proteins, KREPB9 and KREPB10. They are conserved and syntenic in trypanosomatids although KREPB10 is absent in *Trypanosoma vivax* and both are absent in *Leishmania*. Tandem affinity purification (TAP)-tagged KREPB9 and KREPB10 incorporate into ~20S editosomes and/or subcomplexes thereof and preferentially associate with deletion subcomplexes, as do KREPB6, KREPB7, and KREPB8. KREPB10 also associates with editosomes that are isolated via a chimeric endonuclease, KREN1 in KREPB8 RNA interference (RNAi) cells, or MEAT1. The purified complexes have precleaved editing activities and endonuclease cleavage activity that appears to leave a 5' OH on the 3' product. RNAi knockdowns did not affect growth but resulted in relative reductions of both edited and unedited mitochondrial mRNAs. The similarity of KREPB9 and KREPB10 to KREPB6, KREPB7, and KREPB8 suggests they may be accessory factors that affect editing endonuclease activity and as a consequence may affect mitochondrial mRNA stability. KREPB9 and KREPB10, along with KREPB6, KREPB7, and KREPB8, may enable the endonucleases to discriminate among and accurately cleave hundreds of different editing sites and may be involved in the control of differential editing during the life cycle of *T. brucei*.

Most mitochondrial transcripts in trypanosomes undergo uridine (U) insertion and deletion RNA editing in order to form translatable mRNAs. The transcripts are derived from their unusual mitochondrial DNA, also called kinetoplast DNA (kDNA), which is the hallmark of the order and is composed of two types of molecules: maxicircles and minicircles. Tens of maxicircles and thousands of smaller minicircles are joined together to form a single concatenated network within the single mitochondrion of these cells. The editing process is widespread throughout the order Kinetoplastida (29), and its presence among the early diverged Kinetoplastida suggests that its origin is ancient (13). The extent of editing varies among the transcripts within a single organism and among the different kinetoplastid taxa (47). Twelve mRNAs require RNA editing, and nine of these are extensively edited by the insertion of hundreds and deletion of tens of Us in *Trypanosoma brucei*, while editing is less extensive in *Leishmania tarentolae*.

Editing is catalyzed by editosomes, multiprotein complexes that contain a common set of 12 proteins, and three endonucleases that form mutually exclusive editosomes with their KREPB partner proteins (Fig. 1) (10, 34, 48). A partial protein-protein interaction map of the editosome that was generated using a combination of methods revealed a general structural organization that helps illuminate how the coordinated series of catalytic steps occurs during editing (45). Two heterotrimeric subcomplexes with stable protein-protein interactions were identified: the insertion subcomplex KRET2-KREPA1-KREL2 catalyzes guide RNA (gRNA)-directed U addition (by the TUTase KRET2) and ligation (by the ligase KREL2), while the deletion subcomplex KREX2-KREPA2-KREL1 catalyzes gRNA-specified U removal (by the exoUase KREX2) and ligation (by the ligase KREL1). The KREPA1 and KREPA2 proteins directly interact with and enhance the activities of their binding partners (14, 15, 44). They also bind directly to members of a network of interacting OB-fold proteins

and two proteins with degenerate RNase III motifs, KREPB4 and KREPB5. This organized set of 12 common proteins forms the core of the editosomes that interacts with each of the three different endonucleases and their partner proteins, KREN1-KREPB8-KREX1, KREN2-KREPB7, or KREN3-KREPB6. Other complexes and proteins participate in RNA editing and/or other steps in mitochondrial RNA processing (1, 3, 16, 17, 21, 50). The Mitochondrial Editosome-like complex-Associated TUTase 1 (MEAT1) has been shown to associate with editosome proteins, and this protein adds uridines to RNA substrates *in vitro* (5).

Eight of the editosome proteins each have a U1 zinc-finger motif, and they were originally grouped together as KREPB1 to -8 (48). Three of these proteins, initially designated KREPB1, KREPB2, and KREPB3, have been shown to be editing endonucleases and accordingly have been renamed. KREN1 endonuclease cleaves deletion editing sites (ESs), while KREN2 and KREN3 cleave insertion ESs, each with different specificities. Each of these three proteins has a single RNase III motif with key conserved residues that are essential for endonucleolytic cleavage (10, 38, 52). KREPB4 and KREPB5 have a single RNase III motif that

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diverges from typical RNase III motifs, and mutation of conserved residues indicates they are not essential for cleavage (J. Carnes and A. Schnauffer, unpublished). The active sites of RNase III-type endonucleases are formed by two opposing RNase III domains that typically cleave both strands of a double-stranded substrate. Interaction of KREPB4 or KREPB5 with one of the endonucleases may result in a heterodimeric RNase III domain capable of cleavage of only the mRNA strand of the substrate duplex (10). KREPB6, KREPB7, and KREPB8 likely play a role in endonuclease function, since each is exclusively found in KREN3, KREN2, and KREN1 editosomes, respectively.

While the proteins responsible for the catalytic activities of cleavage, U insertion or deletion, and ligation have been identified and characterized, almost nothing is known about the progression of editing from site to site (2) or transition between gRNAs. RNA editing proceeds 3' to 5' but not always precisely in sequential order (19, 27). Each gRNA specifies the editing of several ESs, and extensively edited mRNAs require multiple gRNAs. It is unknown how editosomes switch between insertion and deletion editing, especially in the cases where single gRNAs specify editing of both insertion and deletion ESs.

This article describes two newly discovered B-family editosome proteins, KREPB9 and KREPB10, which have a single N-terminal U1-like zinc finger and like KREPB8 have a WGR motif that may be involved in nucleic acid binding. The composition and activities of KREPB9 and KREPB10 complexes isolated by tandem affinity purification (TAP) tags reveal enrichment in deletion sub-complexes consistent with a preferential physical association for this part of the editosome. Endogenous KREPB10 was also found in complexes that were isolated via tagged MEAT1, a chimeric endonuclease, or KREN1 expressed in cells that repress KREPB8. *In vitro*, the KREPB9 and KREPB10 complexes have typical editosome activities in precleaved assays and atypical cleavage products in endonuclease activity assays. Neither KREPB9 nor KREPB10 appears to be essential to the cells, although RNA interference (RNAi) knockdowns result in an overall decrease in both pre-edited and edited mitochondrial transcripts. Together, the data presented here suggest that KREPB9 and KREPB10 are editosome accessory factors that promote mRNA stability and may affect endonuclease substrate recognition.

## MATERIALS AND METHODS

**TAP tag constructs and purifications.** TAP-tagged wild-type KREPB9 and KREPB10 genes were cloned into the pLEW-MHTAP vector (34), which includes both Myc and His epitopes followed by the typical TAP construct of the calmodulin-binding protein domain, tobacco etch virus (TEV) protease cleavage site, and 2 copies of protein A, creating the plasmids KREPB9-MHTAP and KREPB10-MHTAP. KREPB9 contains an internal restriction site for HindIII that was mutated to allow cloning with HindIII and BglII. Gene fragments were amplified using the following oligonucleotides for KREPB9: 6621 (ATAAAGCTTATGCTCACTCAGGTCCACAAT) to introduce a HindIII site, 6622 (AGTAATAAACGCAAGTTAGATTTCGTTGGGA) and 6623 (TCCCAACGAATCTAAACTTGCGTTATTACT) for removal of an internal HindIII site, and 6624 (ATAA GATCTGCAAAAAGGTGCTTTCC) to introduce the BglII site for cloning into the pLEW-MHTAP vector. Gene fragments were amplified using the following oligonucleotides for KREPB10: 6625 (ATAAAGCTTATGATGTACCGGTGGA) to introduce the HindIII site and 6626 (ATAAGATCTCGCTGCGCCTATTAATAC) to introduce the BglII site for cloning into the pLEW-MHTAP vector. Chimeric endonuclease construction is described in the supplemental material. For each construct, the plasmid was

NotI digested and transfected into 29.13 cells, followed by selection with phleomycin to generate the cell lines KREPB9-TAP and KREPB10-TAP.

Editosome complexes were isolated from  $\sim 6 \times 10^{10}$  cells for KREPB9-TAP and KREPB10-TAP by lysis with Triton X-100 and clarification by centrifugation, followed by sequential IgG and calmodulin affinity chromatography as previously described (34, 41). In parallel, complexes were purified via IgG affinity from  $4 \times 10^{10}$  KREPB9-TAP or KREPB10-TAP cells for glycerol gradient fractionation. TEV eluates were loaded onto 11 ml 10% to 30% (vol/vol) glycerol gradients as previously described (10). Briefly, gradients were centrifuged at 38,000 rpm for 12 h at 4°C in a Beckman SW40 rotor, and 500  $\mu$ l fractions were collected from the top of the gradients, flash frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$  until further analysis.

**Mass spectrometry.** Protein complexes eluted from calmodulin columns were denatured with 8 M urea, diluted 1:8, and digested in solution with trypsin. The resulting peptides were fractionated and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described previously (35, 37).

**Immunofluorescence microscopy.** Noninduced and induced cells ( $\sim 5 \times 10^5$ ) were pelleted by centrifugation, washed, and fixed with 4% formaldehyde. The fixed cells were permeabilized with Triton X-100 and then blocked with fetal bovine serum (FBS) for 1 h. The cells were then incubated with two primary antibodies: 1:200 rabbit polyclonal antibody to Myc (Sigma) to visualize the MHTAP-tagged protein and 1:4 mouse monoclonal antibody to heat shock protein 70 (HSP70) (mAB78) (39) as a mitochondrial control. Secondary antibodies used were 1:500 goat anti-rabbit-fluorescein isothiocyanate (FITC) (Sigma) and 1:400 goat anti-mouse-TxRed (Invitrogen). For 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining, cells were treated with 50  $\mu$ l of 1  $\mu$ g/ml DAPI to visualize DNA. A phase-contrast image of the cells and their fluorescence was captured with a Nikon fluorescence microscope equipped with camera and the appropriate filters.

**Western, adenylation, and SDS-PAGE analyses.** Glycerol gradient fractions (30  $\mu$ l) were resolved by 10% SDS-PAGE (Bio-Rad), and transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P) (see Fig. 3A and B). The membrane was blocked in 10% nonfat milk powder in PBST (10 mM phosphate buffer, pH 7.2, 150 mM NaCl, 0.1% Tween 20) overnight at 4°C. The membrane was washed with PBST and probed with a cocktail of monoclonal antibodies (MAbs) specific for KREPA1, KREPA2, KREL1, and KREPA3 (37) in 5% nonfat milk powder in PBST for 2 h at room temperature. The membrane was then washed with PBST and incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (Bio-Rad) in 1% nonfat milk powder in PBST. ECL enhanced chemiluminescence (Pierce) was used to visualize antigens. To obtain the results shown in Fig. 3C, TAP-purified protein eluates were analyzed using polyclonal anti-MEAT1 and monoclonal anti-KREPA1, -KREPA2, -KREL1, and -KREPA3 antibodies simultaneously using the Odyssey system. The separated proteins were blotted on Immobilon-FL membranes and blocked in LiCor blocking buffer at 4°C overnight. The primary antibodies were diluted in 1:1 blocking buffer and PBST. Membranes were washed four times in PBST for 5 min each. The secondary antibodies were diluted 1:15,000 in 1:1 blocking buffer and PBST in the dark. Membranes were washed four times in PBST for 5 min each, dried, and scanned using the LiCor Odyssey system. Adenylation of RNA editing ligases (KREL1 and KREL2) was assayed as described previously (43). The reaction products were separated by 10% SDS-PAGE (Bio-Rad), and the radiolabeled proteins were visualized using a PhosphorImager (GE Healthcare). SYPRO ruby staining of complexes fractionated in 10% SDS-PAGE (Bio-Rad) was performed according to the manufacturer's protocol (Molecular Probes).

**RNA editing assays.** KREPB9-TAP and KREPB10-TAP were assayed using *in vitro* editing assays. Precleaved insertion and deletion activities were assayed as previously described by using 5'-labeled 5'CL18 and 3'CL13pp with gPCA6-2A RNAs (22) and 5'-labeled U5 5'CL and U5 3'CL with gA6[14]PC-del RNAs (23), respectively. Reaction products



were resolved on 11% acrylamide with 7 M urea gels and visualized using a PhosphorImager (GE Healthcare). Cleavage assays were based on full-round insertion and deletion assays using ATPase synthase subunit 6 (A6) substrate RNA and were modified to enhance formation of the endonucleolytic cleavage product as described previously (8, 49). Insertion cleavage assays used A6-eES1 pre-mRNA and gA6[14] gRNA. Deletion cleavage assays used the A6short/TAG.1 pre-mRNA and D34 gRNA, which is a derivative of D33' (12).

**RNA interference cell lines.** RNA interference (RNAi) constructs were generated for expressing double-stranded RNA under tetracycline (Tet) regulation for KREPB9 and KREPB10 by inserting fragments of the gene into the RNAi vector pQuadra (24). A 535-bp fragment corresponding to 594–1129 nucleotide region of the KREPB9 coding sequence resulted in knockdown of expression of the KREPB9 mRNA. A 461-nucleotide (nt) fragment corresponding to the 292–753 nucleotide region of the KREPB10 coding sequence resulted in knockdown in the expression level of the KREPB10 mRNA. The 535-bp KREPB9 gene fragment was amplified using the oligonucleotides 7727 (ATACCAATGTGATGGTGC TTTAACTGGCGATTGTG) and 7728 (ATACCAATGTGATGGGTACA ACCGCTTCCCTTTCA). The 461-bp KREPB10 gene fragment was amplified using the oligonucleotides 7229 (ATACCAATGTGATGGCGCTC GGAGTTTCTTCAATC) and 7230 (ATACCAATGTGATGGCCACAAT GAGACGTGACACC). These gene fragments were ligated to a short hairpin loop and cloned into the vector using the BstXI restriction sites and enzyme (24). RNAi cell lines were generated in 29.13 cells by transfection with 10 µg of NotI-linearized pQuadra construct, using a published procedure (51). Transfectants were selected in the presence of 15 µg/ml G418, 25 µg/ml hygromycin, and 2.5 µg/ml phleomycin. The resultant stable cell line was designated KREPB9 RNAi and KREPB10 RNAi. RNAi was induced with 1 µg/ml tetracycline, and the uninduced and induced cells were counted daily to obtain growth curves. Cells were maintained between  $2 \times 10^6$  and  $3.5 \times 10^7$  cells/ml.

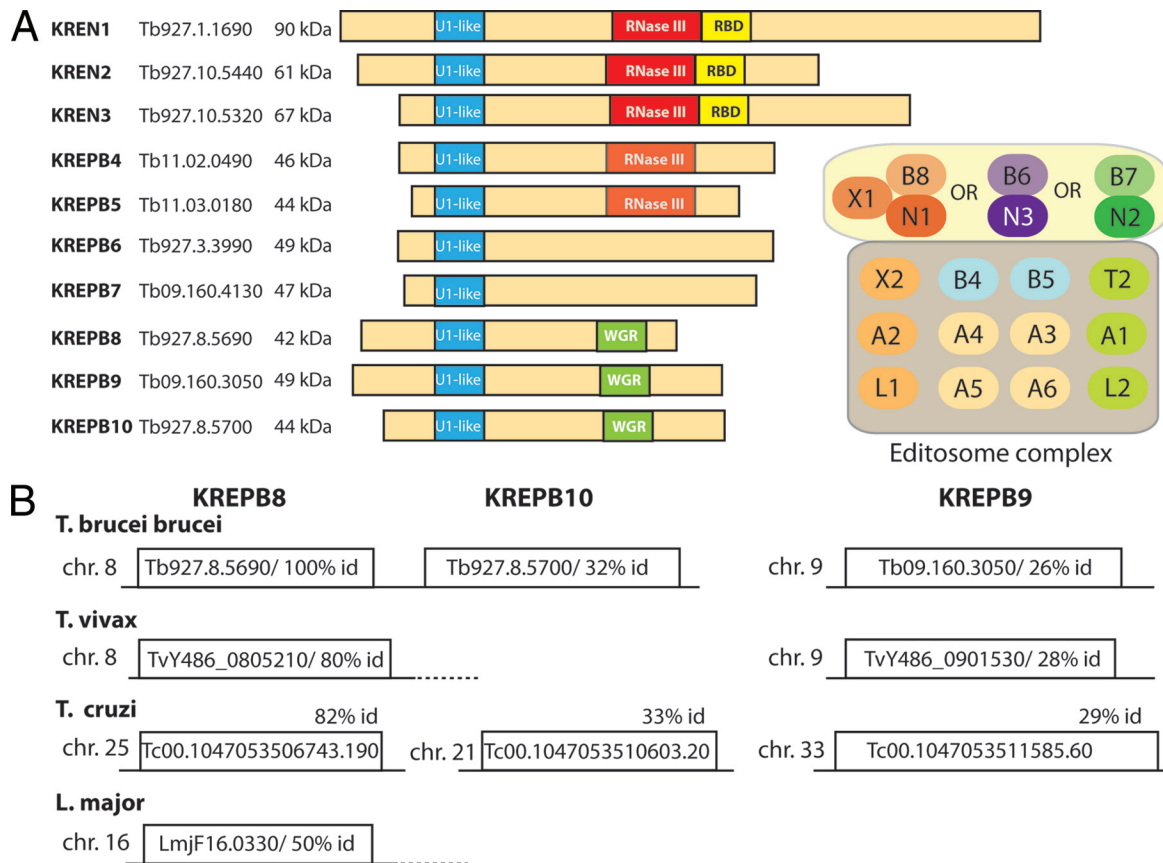
**RNA isolation and real-time RT-PCR (qPCR).** Quantitative real-time reverse transcriptase PCR (quantitative RT-PCR [qPCR]) was carried out to assess preedited and edited mRNA levels essentially as described previously (8). Briefly, total RNA was isolated from  $1 \times 10^8$  cells grown in the presence or absence of tetracycline for 4 days using TriPure reagent as described by the manufacturer (Roche). Ten micrograms of total RNA was treated with DNase I using the DNA-free kit (Ambion). The integrity of the DNase-treated RNA was confirmed using an RNA nanochip on a Bioanalyzer instrument (Agilent Technologies). The cDNA templates for real-time PCR were reverse transcribed from 4.5 µg of RNA using random hexamers and TaqMan reverse transcription reagents (Applied Biosystems) in a 30-µl reaction mixture. Control reactions without reverse transcriptase were used to confirm the absence of contaminating genomic DNA. Primers for real-time PCR were designed using the ABI Primer Express v2.0 software program. The sequences of all the primers for each preedited and edited mRNA used were described previously (8), except KREPB9 and KREPB10 forward (5'-CGCTGAGTAACTTGCGTTTGG-3') and reverse (5'-CGCACCTTCATTGGCATGT-3') and KREPB9 forward (5'-TCCGAATGCCGATGCTAGAGA-3') and reverse (5'-CGCACC TTCATTGGCATGT-3'). The cDNA reaction mixtures were diluted between 1:7 and 1:50 and 2.5 µl of this cDNA template (or RT-free control) was used in 25-µl reaction mixtures containing 12.5 µl of SsoFast EvaGreen supermix with low ROX (Bio-Rad) and 5 µl (each) of 1.5 µM forward and reverse primers in 96-well plates using the ABI Prism 7500 sequence detection system (Applied Biosystems). Amplification conditions for all reactions were 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. Each reaction was carried out in triplicate. Thermal dissociation curves confirmed that the PCR generated a single amplicon. PCR efficiencies were calculated using LinReg software analyzing the slope of the PCR amplification. Relative changes for target RNAs were determined after normalization to 18S rRNA and were expressed as fold change with respect to results for control cells. Two separate experiments were combined for the reported results.

## RESULTS

**Identification of KREPB9 and KREPB10.** KREPB9 and KREPB10 were identified by BLAST searches in GeneDB to find *T. brucei* protein sequences related to that of KREPB8. Four sequences with significant similarity to that of KREPB8 were identified: those of KREPB10, KREPB9, KREPB7, and KREPB6, in order of the highest probability score. All five proteins have a single N-terminal U1-like zinc finger that is characteristic of the B-family editosome proteins (Fig. 1). In addition, KREPB8, KREPB9, and KREPB10 share a motif with significant similarity to the tryptophan-, glycine-, and arginine-rich (WGR) domain described in Pfam (PF05406). This motif was identified in KREPB8, KREPB9, and KREPB10 by analyzing a set of 52 *Trypanosoma* B-family proteins, including the editing endonucleases (see Table S1 in the supplemental material) using the MEME (Multiple EM for Motif Elucidation) software program (6). The precise function of the WGR domain is uncertain, but a role in nucleic acid binding is implied.

**Sequence and conservation of syntenic arrangement of KREPB8, KREPB9, and KREPB10 among trypanosomatids.** KREPB10 has 32% amino acid sequence identity and 48% similarity to KREPB8, and the corresponding genes are adjacent on chromosome 8, while KREPB9 has 26% identity and 42% similarity to KREPB8 and is encoded on chromosome 9 (Fig. 1). Other trypanosomatids retain KREPB9 and KREPB10 with similar amounts of sequence conservation and the same syntenic arrangement. However, KREPB10 is absent from the syntenic site in *Trypanosoma vivax*, and no sequence similarity to KREPB10 was found in the intergenic space between the KREPB8 gene and the 3' adjacent gene (Tb927.8.5710, recombination initiation protein 1). The most recent *T. vivax* genome sequence has 6-fold coverage (completed March 2006), and the coverage of KREPB8 and the surrounding regions appears sufficient to detect KREPB10, if it were present. In addition, the *T. vivax* KREPB8 sequence is more diverged from that of *T. brucei* than from that of the more phylogenetically diverged *T. cruzi*, which retains both KREPB9 and KREPB10. Thus, KREPB10 is absent from *T. vivax*. KREPB8 and KREPB10 are encoded on different chromosomes in *T. cruzi*, rather than adjacent as in *T. brucei*. In contrast, both KREPB9 and KREPB10 are absent from *Leishmania*. The KREPB9 gene and the ortholog of the next 3' gene in *T. brucei* (Tb09.160.3060) are absent from *Leishmania* in the region that retains the orthologs of the flanking genes. Specifically, *Leishmania* retains the ortholog of Tb09.160.3020 that is directly 5' to the KREPB9 gene in *T. brucei* and the ortholog of Tb09.160.3090 that is directly 3' to Tb09.160.3060, essentially replacing two genes from *T. brucei* (KREPB9 and Tb09.160.3060) with a single gene in *Leishmania*. In the genomes where the KREPB10 gene was not identified, the syntenic region that is flanked by the KREPB8 and Tb927.8.5710 orthologs is relatively small and is insufficient to contain even a highly diverged full-length KREPB10 gene.

**KREPB9 and KREPB10 are mitochondrial proteins.** Transgenic cell lines with tetracycline-regulatable expression of C-terminal TAP-tagged versions of KREPB9 and KREPB10 were used to determine protein localizations and complex associations. The TAP tag construct includes Myc and His epitopes flanked by a calmodulin binding protein domain and C-terminal tandem protein A domains. Immunofluorescence analysis of permeabilized fixed cells using a polyclonal anti-Myc primary antibody and goat anti-rabbit secondary antibody conjugated to FITC revealed mor-



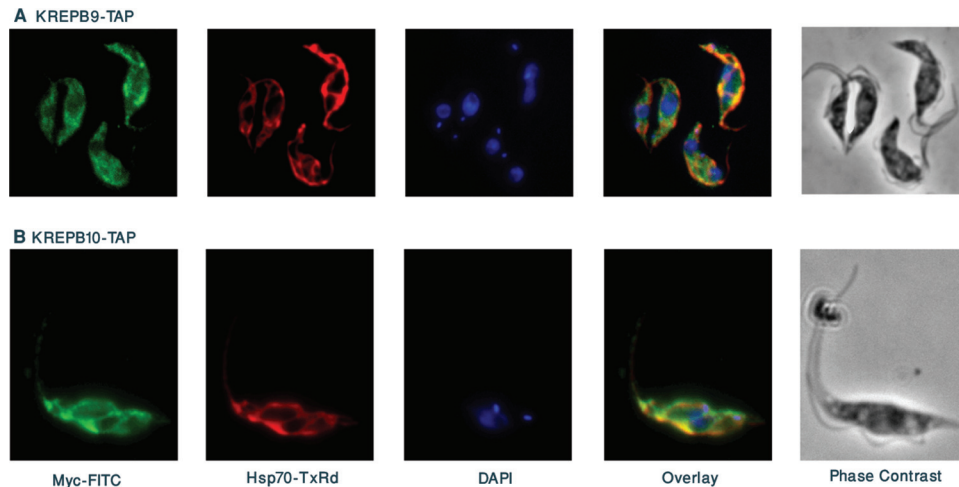
**FIG 1** KREPB9 and KREPB10 editosome proteins. (A) Diagram of editosome proteins aligned by the U1-like zinc finger (blue) with other domains, RNase III (red), double-stranded RNA binding motif (RBD) (yellow), degenerate RNase III (orange), and WGR (green), indicated. The inset shows a diagram of editosome complex organization using abbreviations outlined in Table 1, highlighting the set of 12 common editosome proteins, as well as those uniquely associating with each endonuclease. (B) The syntenic relationship of KREPB9 and KREPB10 within some members of the Kinetoplastida. The percent identity (id) to *T. brucei* KREPB8 and chromosome location (chr.) are indicated.

phology consistent with the procyclic-form mitochondrion (Fig. 2). The fluorescent signal for the TAP-tagged proteins colocalized with the fluorescent signal from mitochondrial HSP70-specific monoclonal antibody detected with a goat anti-mouse secondary antibody conjugated to Texas Red. KREPB9 had a weaker signal than KREPB10, possibly implying a smaller amount of protein. KREPB9 was previously reported to have mitochondrial localization using a green fluorescent protein (GFP) fusion protein (53). Thus, both KREPB9 and KREPB10 are mitochondrial proteins.

**KREPB9 and KREPB10 form distinct associations with editosome proteins.** Proteins associated with KREPB9 or KREPB10 were identified by affinity purifying the TAP-tagged complexes. Tagged KREPB9 or KREPB10 complexes bound to IgG-Sepharose affinity columns were eluted by TEV protease cleavage and fractionated on 10 to 30% glycerol gradients for 12 h, revealing both ~10S and ~20S complexes. Gradient fractions analyzed by Western blotting using a mixture of the monoclonal antibodies for KREL1, KREPA1, KREPA2, and KREPA3 revealed that these four editosome proteins were associated with the tagged KREPB9 and KREPB10 complexes (Fig. 3). The tagged complexes contained proportionally more KREPA2 and KREL1, less KREPA3, and dramatically less KREPA1 than untagged mitochondrial ~20S complexes. The KREL1 autoadenylation activity and the typical weaker KREL2 signal (43) essentially mirrored the Western detec-

tion of KREL1 except that it was detected only in KREPB9 fractions 5 and 7. A substantial proportion of the complexes sedimented at ~10S (fractions 9 to 13) and exhibited a relative preponderance of KREPA2 and KREL1, which is characteristic of proteins associated with the deletion subcomplex. Some complexes sedimented in fractions 15 to 19, where ~20S editosomes would be in a 12-h gradient, but had proportionally less KREPA1 and KREPA3 than ~20S controls.

TEV eluates of KREPB9-TAP and KREPB10-TAP complexes were further purified by calmodulin affinity chromatography and examined by Western analysis and mass spectrometry. Western analysis with the monoclonal antibody mixture for KREL1, KREPA1, KREPA2, and KREPA3 was similar to the glycerol gradient results, although the recovery from the calmodulin columns was low, as is often the case (Fig. 3C). Less protein was recovered in the calmodulin binding protein eluates (CBE), but the relative proportions of the proteins were similar to those of the TEV eluates. In addition, Western analysis with a polyclonal antibody to the mitochondrial editosome-like complex-associated TUTase (MEAT1) revealed the presence of this protein in the purified KREPB9, KREPB10, and KREPB5 complexes (5). SYPRO ruby-stained SDS-PAGE gels of the calmodulin column eluates revealed profiles that were similar to each other's and to those of editing complexes, albeit with some differences in the protein ratios.



**FIG 2** Mitochondrial localization of KREPB9 and KREPB10. Expression of tagged KREPB9 and KREPB10 was induced for 2 days with 1  $\mu\text{g}/\text{ml}$  tetracycline and detected using a polyclonal anti-Myc antibody. Mitochondrial HSP70 was used as a positive control for mitochondrial localization. Nuclear and kinetoplast DNAs were visualized with DAPI.

KREPB5-TAP was used as a positive control for affinity purification of editosome complexes because its purified complexes contain essentially all of the editosome proteins, including the endonucleases. Mass spectrometric analysis of CBEs and SDS-PAGE gel slices of KREPB10 identified all editosome proteins except for KREPB6, KREPB7, and KREPB9. Only a single KREPB8 peptide was detected. Analysis of KREPB9 material, of which much less was recovered, resulted in detection of the deletion subcomplex proteins KREX2, KREPA2, and KREL1. The KREPA3, KREPA4, KREPA5, and KREPA6 proteins, as well as the KREPB4 and KREPB5 proteins with degenerate RNase III motifs, were identified. The insertion subcomplex proteins were not detected, nor were the endonucleases, the deletion endonuclease editosome-specific protein KREX1, or the B proteins that are typically associated with the endonucleases (KREPB6, KREPB7, or KREPB8). Of the related KREPB6, KREPB7, KREPB8, KREPB9, and KREPB10 proteins, only KREPB9 was identified (Table 1).

**Endogenous KREPB10 detected by mass spectrometry.** Numerous mass spectrometric analyses of purified mitochondria and purified editosomes were examined for peptides corresponding to either endogenous KREPB9 or KREPB10. Neither KREPB9 nor KREPB10 was detected in a thorough examination of the mitochondrial proteome, including 946 independent experiments in which other editosome proteins were detected 603 times (36). In 9 additional independent mass spectrometry analyses of purified mitochondria, editosome proteins were detected 266 times, but peptides corresponding to KREPB9 or KREPB10 were not found. Similarly, peptides corresponding to KREPB9 were not detected in any experiments that examined purified editosomes. However, among 363 independent analyses of purified editosomes, peptides corresponding to KREPB10 were detected in 3 experiments: editosomes isolated via TAP-tagged MEAT1, KREN2-KREN1 chimeric endonuclease or KREN1 editosomes isolated from cells that express KREPB8 RNAi. Excluding peptides corresponding to the tagged bait protein, a total of 7,869 peptides of editosome proteins have been detected, with the average number of peptides detected for one of the editosome proteins being  $\sim 342$  (see Table S2 in the supplemental material). Considering that only 15 peptides from

KREPB10 were detected in these experiments, it appears to be a low-abundance protein.

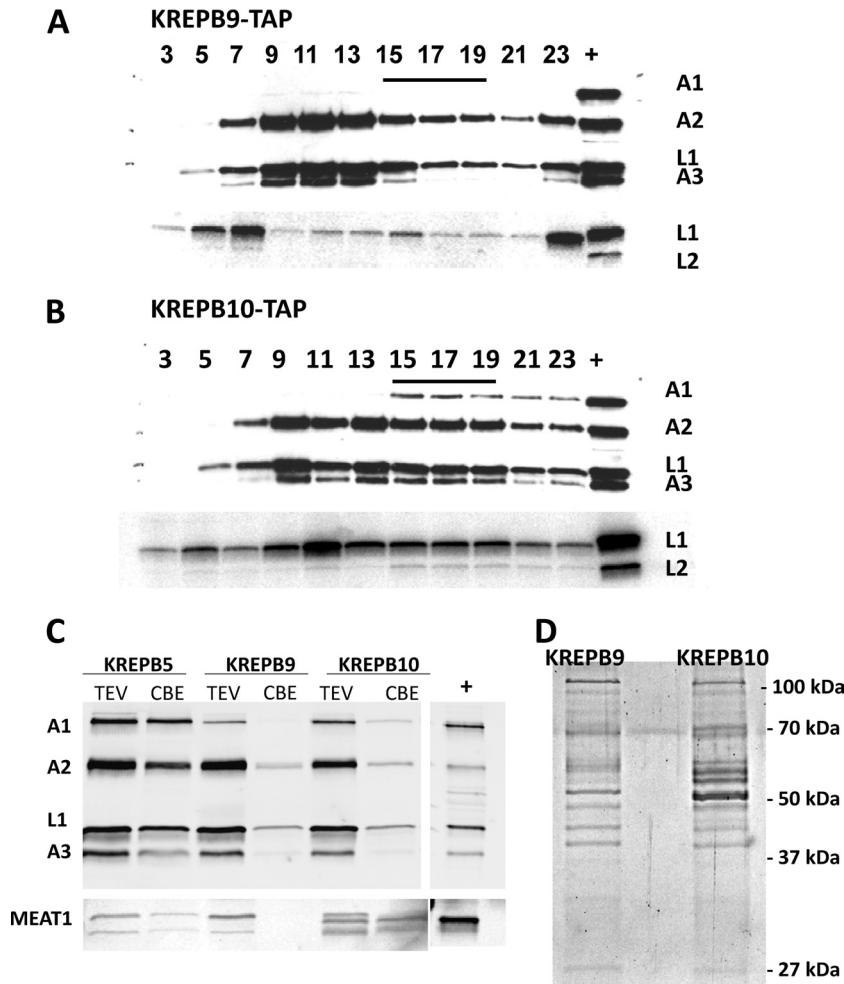
**TAP-isolated editosomes that contain endogenous KREPB10.**

The unique nature of the three different TAP-purified editosomes that contain endogenous KREPB10 may shed light on the role of KREPB10 (see the supplemental material). KREPB10 was identified by mass spectrometric analysis of complexes that were purified via TAP-tagged MEAT1 (see Fig. S1 in the supplemental material). All other proteins that are stably associated with editosomes were also detected in these complexes (see Table S3), while previously published results detected only some of these (5). However, MEAT1 was not detected in numerous experiments that analyzed editosomes that were purified via various TAP-tagged editosome proteins, even though MEAT1 was readily detected in purified mitochondria and other TAP-purified mitochondrial samples (see Table S2). Thus, although MEAT1 is reasonably abundant, it is not a stable component of editosomes. Peptides corresponding to endogenous KREPB10 were also detected by mass spectrometry of complexes that were isolated from cells that constitutively express TAP-tagged KREN1 and that decrease KREPB8 expression by Tet-induced RNAi (see Table S3) (18). Thus, KREPB10 can be associated with editosomes that lack KREPB8. Perhaps KREPB10 and KREPB8 compete for association with editosomes since they are similar. Furthermore, KREPB10 was detected in editosomes that were isolated via a tagged chimeric KREN2-KREN1 protein in which the N-terminal U1 zinc finger region of KREN2 replaced that of KREN1. Western analyses showed that the chimeric endonuclease was incorporated into the complexes (see Fig. S2B). Mass spectrometric analyses of these tagged chimeric complexes also detected peptides for KREX1 exoUase and KREPB8 (see Table S4). This result is notable, since KREX1 and KREPB8 have been found associated only with tagged KREN1 editosomes. The replacement of the N-terminal region in the chimeric endonuclease may have altered the editosome such that association with KREPB10 is promoted.

**Enzymatic activities of KREPB9 and KREPB10 complexes.**

The catalytic activities of the tagged KREPB9 and KREPB10 complexes were evaluated using precleaved insertion-and-deletion as-





**FIG 3** TAP-tagged KREPB9 and KREPB10 editosomes. (A) Western analyses of TAP-tag purified KREPB9 complexes fractionated on a 10 to 30% glycerol gradient. Tagged complexes were enriched from whole-cell lysates by IgG affinity chromatography and TEV elution, and fractionated TEV eluate was analyzed using a mixture of monoclonal antibodies specific for KREPA1, KREPA2, KREL1, and KREPA3 (A1, A2, L1, and A3; top panel). The positive control (+) is a ~20S fraction from purified mitochondria, and the black bar shows the location of the ~20S portion of the gradient. KREL1 and KREL2 were detected by autoadenylation (bottom panel). (B) Similar analysis of KREPB10 complexes. (C) Western analysis of TEV and calmodulin binding eluates (CBE) using the four-editosome antibody mixture as above (top panel) and a polyclonal antibody to MEAT1 (bottom panel). (D) SYPRO ruby-stained 10% SDS-PAGE gel of the calmodulin binding eluates of KREPB9 and KREPB10.

says (Fig. 4A and B). Both the TEV eluates and CBE of KREPB9 and KREPB10 had robust precleaved deletion activity, including the stepwise exoUase removal of Us, ligation of the input substrate, and ligation of the substrate from which Us were removed. TEV eluates of both tagged complexes exhibited precleaved insertion activity, but CBE of KREPB10 was particularly weak (evident from the higher proportion of ligated to edited product), and CBE of KREPB9 lacked U addition activity. Some of the low activity reflects the limited amount of material and the narrow dynamic range of the assay. Overall, these tagged complexes have all of the precleaved activities characteristic of editosomes.

Endonuclease cleavage assays were performed on the TEV eluates and CBE purified tagged KREPB9 and KREPB10 complexes to assess their activity at deletion and insertion sites (Fig. 4C and D). A few similar-size 3' cleavage products were generated rather than a single prominent cleavage product as is seen with total ~20S complexes ("20S +g" lane). Typically, endonuclease cleavage products have a 5' phosphate, which leads to an intermediate

position relative to bands in the T1 and hydroxyl (OH) ladders, which have a 5' hydroxyl (40, 46). The relative positions of the cleavage products produced by the KREPB9 and KREPB10 complexes were more similar to those of the T1 and hydroxyl products than to those from the total ~20S complexes, which position between the bands of the OH ladder. This result suggests that the cleavage products lack this 5' phosphate. In addition, the relative intensity of these products is increased in CBE over that in TEV eluates.

**RNAi knockdown of KREPB9 or KREPB10 does not cause a growth defect.** RNAi knockdown of either KREPB9 or KREPB10 did not inhibit growth of procyclic forms (Fig. 5A) but affected the levels of mitochondrial mRNAs. Quantitative PCR analysis of pre-edited and edited mitochondrial mRNA was performed with total RNA collected following 4 days of tetracycline-induced RNAi knockdown (Fig. 5B and C). Induction of RNA interference in either cell line resulted in a reduction of KREPB9 or KREPB10 mRNA by 29% and 43%, respectively, compared to the level in cells in which RNAi was not induced. Both edited and preedited

**TABLE 1** Protein composition of the KREPB9-TAP and KREPB10-TAP complexes as determined by mass spectrometry

| Gene ID <sup>a</sup> | Protein        | Role         | Peptides detected via <sup>b</sup> : |            | Note                           |
|----------------------|----------------|--------------|--------------------------------------|------------|--------------------------------|
|                      |                |              | KREPB10 TAP                          | KREPB9 TAP |                                |
| Tb927.1.1690         | <u>KREN1</u>   | Endonuclease | +                                    |            | Deletion cleavage              |
| Tb927.10.5440        | <u>KREN2</u>   | Endonuclease | +                                    |            | Insertion cleavage             |
| Tb927.10.5320        | <u>KREN3</u>   | Endonuclease | *                                    |            | COII insertion cleavage        |
| Tb927.3.3990         | <u>KREPB6</u>  | Interaction  |                                      |            | KREN3 accessory protein        |
| Tb09.160.4130        | <u>KREPB7</u>  | Interaction  |                                      |            | KREN2 accessory protein        |
| Tb927.8.5690         | <u>KREPB8</u>  | Interaction  | *                                    |            | KREN1 accessory protein        |
| Tb09.160.3050        | <u>KREPB9</u>  | Interaction  |                                      | +          | Endonuclease accessory protein |
| Tb927.8.5700         | <u>KREPB10</u> | Interaction  | +                                    |            | Endonuclease accessory protein |
| Tb927.7.1070         | <u>KREX1</u>   | Exonuclease  | +                                    |            |                                |
| Tb10.6k15.2310       | <u>KREPA2</u>  | Interaction  | +                                    | +          | Deletion subcomplex            |
| Tb10.70.3850         | <u>KREX2</u>   | Exonuclease  | +                                    | +          | Deletion subcomplex            |
| Tb09.160.2970        | <u>KREL1</u>   | Ligase       | +                                    | +          | Deletion subcomplex            |
| Tb927.2.2470         | <u>KREPA1</u>  | Interaction  | +                                    |            | Insertion subcomplex           |
| Tb927.7.1550         | <u>KRET2</u>   | TUTase       | +                                    |            | Insertion subcomplex           |
| Tb927.1.3030         | <u>KREL2</u>   | Ligase       | +                                    |            | Insertion subcomplex           |
| Tb927.8.620          | <u>KREPA3</u>  | Interaction  | +                                    | +          |                                |
| Tb10.70.2100         | <u>KREPA4</u>  | Interaction  | +                                    | +          |                                |
| Tb927.8.680          | <u>KREPA5</u>  | Interaction  | +                                    | +          |                                |
| Tb927.10.5120        | <u>KREPA6</u>  | Interaction  | *                                    | +          |                                |
| Tb11.02.0490         | <u>KREPB4</u>  | Interaction  | +                                    | +          |                                |
| Tb11.03.0180         | <u>KREPB5</u>  | Interaction  | +                                    | +          |                                |
| Tb927.1.1330         | <u>MEAT1</u>   | TUTase       |                                      |            |                                |

<sup>a</sup> Gene identifier.

<sup>b</sup> “+” indicates that protein was detected by at least 2 tryptic peptides; “\*” indicates that the protein was detected by a single peptide. Blank boxes indicate that the protein was not detected. Shorthand names for editosome proteins are indicated by the underlined part of each full name.

transcripts of A6, COIII, and CYb were analyzed, as well as the never-edited COI. An overall decrease in the amount of mitochondrial RNA was observed upon induction of RNAi of KREPB9 and KREPB10, with a more notable affect with the latter, which also has the higher level of target RNA reduction. This is unlike the effect of knockdown of other editosome proteins, where only the abundance of the edited RNAs was reduced (8, 9, 14, 20, 49).

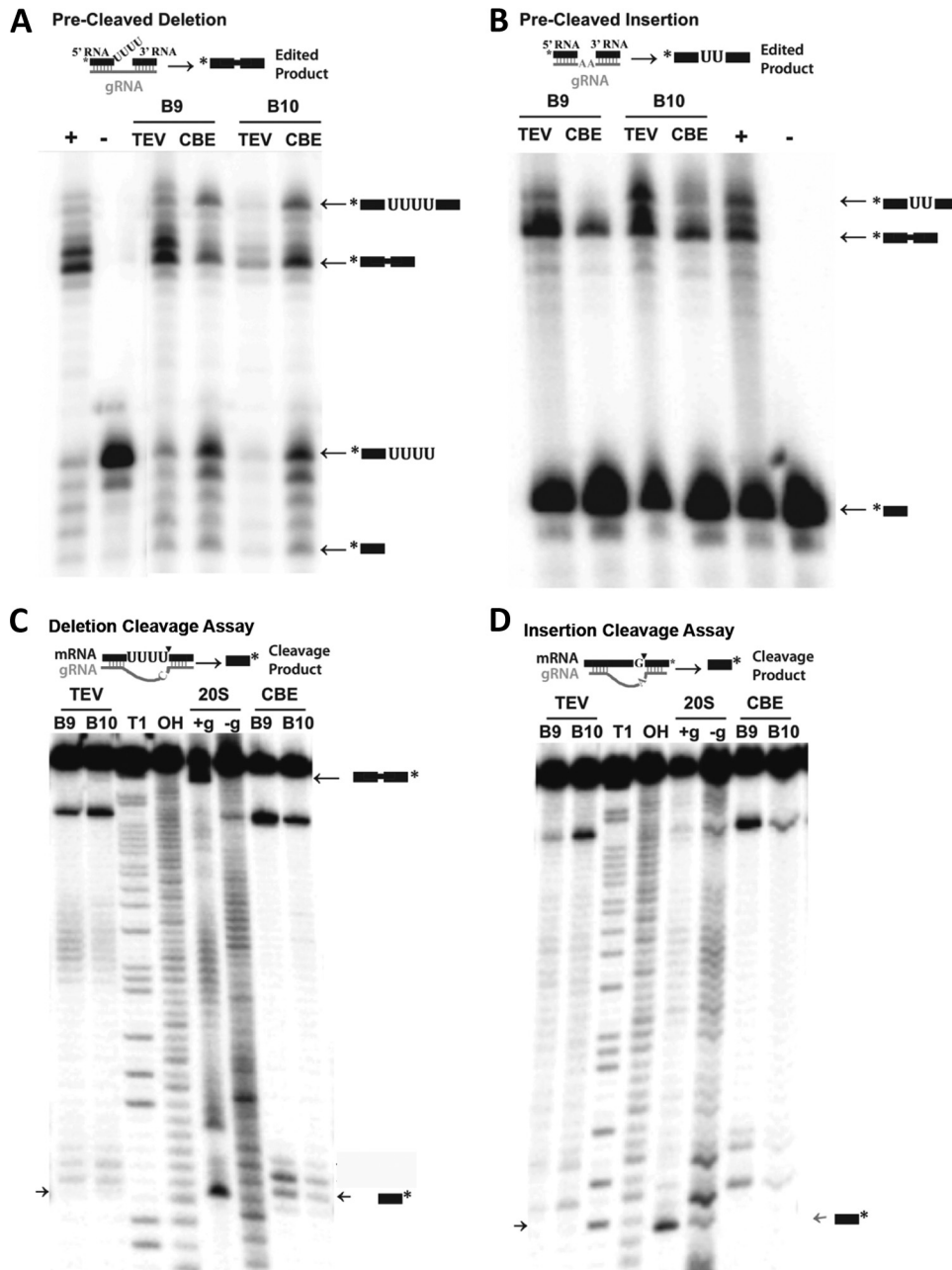
## DISCUSSION

This article describes two new RNA editing proteins, designated Kinetoplastid RNA Editing Proteins B9 and B10 (KREPB9 and KREPB10). They have U1-like zinc finger motifs, as do eight other related editosome proteins, but also have a WGR motif, as does the closely related KREPB8 protein. These three proteins are conserved among the brucei-group trypanosomes and *T. cruzi*, but KREPB9 is absent in *T. vivax*, and both KREPB9 and KREPB10 are absent in *Leishmania*. Tagged KREPB9 and KREPB10 associate *in vivo* with editosomes. The TAP tag purified editosomes have typical precleaved insertion and deletion editing activities and atypical endonuclease activities. KREPB9 and KREPB10 appear to be low-abundance proteins based on the low frequency of detecting their peptides in samples from numerous independent preparations of isolated mitochondria and editosomes. Indeed, endogenous KREPB10 was detected only in complexes purified by tagged MEAT1, tagged KREN1 (from cells that express KREPB8 RNAi),

or tagged chimeric KREN2-KREN1 endonuclease. KREPB9 and KREPB10 may not be essential in procyclic forms, since growth was unaffected by RNAi knockdown; however, the level of knockdown was low. Knockdown did result in a relative decrease in mitochondrial mRNA abundance, particularly with KREPB10. This result mirrors RNAi knockdown of MEAT1, which in contrast led to a relative increase in mitochondrial RNA abundance, suggesting that roles of these proteins may be antagonistic *in vivo*. The KREPB6 to -B10 proteins preferentially interact with the deletion subcomplex, and circumstantial evidence suggests they interact directly or indirectly with editing endonucleases. The low abundance of KREPB9 and KREPB10, the differential presence of these genes in different species, and the potential lack of essentiality suggest that they are accessory proteins that transiently interact with editosomes. Their specific functions are uncertain, but they may affect editosome endonuclease specificity and consequently mitochondrial mRNA abundance.

**Composition of the KREPB9 and KREPB10 complexes.** Purifications via TAP-tagged KREPB9 and KREPB10 each resulted in the isolation of both ~10S and ~20S complexes, although tagged KREPB9 predominantly results in ~10S complexes. Note that tagged KREPB10 pulled down complexes with KREN1, KREN2, and the occasional peptide for KREN3 and KREPB8. The isolation of each type of endonuclease indicates that KREPB10 has a

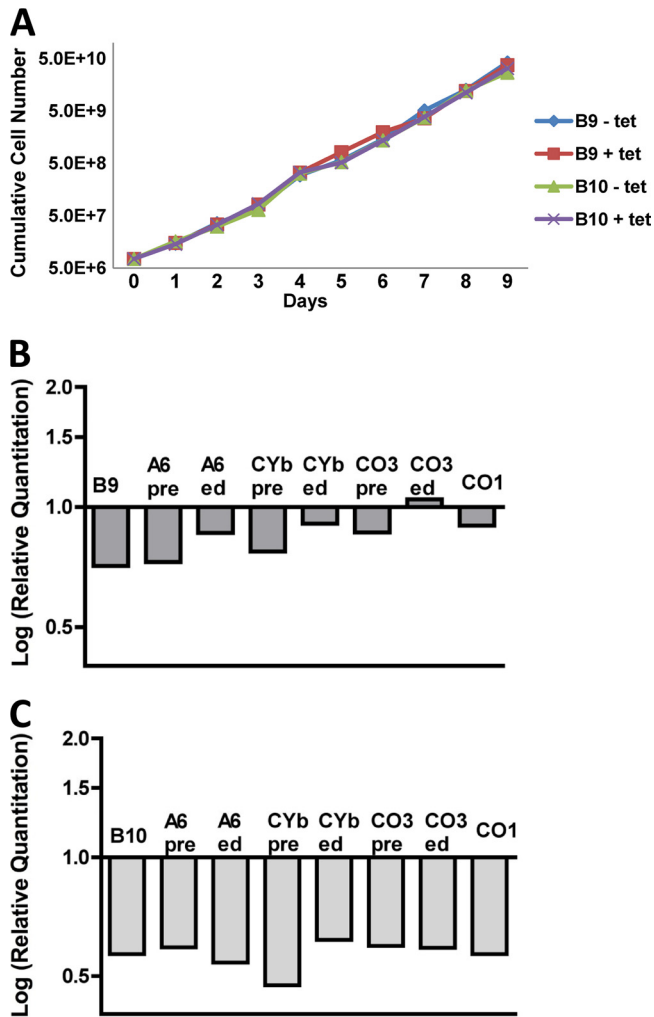




**FIG 4** *In vitro* insertion and deletion editing assays analyzing purified KREPB9 and KREPB10 complexes. Both TEV and CBE were analyzed for precleaved deletion (A) or insertion (B) activities, as well as endonucleolytic cleavage of a deletion (C) or insertion (D) substrate. In each assay, an ~20S fraction from purified mitochondria is used as a positive control (indicated by “+” or 20S). RNA substrates and products are shown schematically, with the asterisk indicating radiolabel and the wedge indicating a typical cleavage site. Typical cleavage products in panels C and D are present in the 20S control with guide RNA (+g) but not in its absence (–g) and are denoted by arrows. Reference ladders were produced by digestion with T1 nuclease (T1) or alkaline hydrolysis (OH).

broader association with editosomes than KREPB6 to -B8. The generation of subcomplexes may result from the overexpression of the TAP-tagged proteins. However, this overexpression did not impact growth or substantially alter editosome function. The ~10S complexes contain the deletion subcomplex proteins, namely, the exoUase KREX2, the RNA ligase KREL1, and their binding partner protein, KREPA2, as shown by a combination of Western, enzyme activity, and mass spectrometric analyses. This result is similar to the ~10S complexes obtained with TAP-tagged

KREPB6, KREPB7, or KREPB8 (10). This implies that all five related proteins have structurally similar associations with the editosome. In contrast, ~10S complexes obtained with TAP-tagged KREN1, KREN2, and KREN3 endonucleases contain the insertion subcomplex (34), as do ~10S complexes isolated with tagged chimeric KREN2-KREN1. Thus, there is a complementary relationship in which KREN1, KREN2, and KREN3 physically associate with the insertion subcomplex and KREPB6 to -B10 associate with the deletion subcomplex.



**FIG 5** RNAi knockdown of KREPB9 and KREPB10. (A) Cumulative growth of cells in which RNAi targeting either KREPB9 or KREPB10 was induced (+tet) or uninduced (–tet) is shown. The x axis is the number of days after RNAi induction. (B and C) Real-time PCR analysis comparing total RNA from cells in which RNAi targeting KREPB9 (B) or KREPB10 (C) was either induced with tetracycline or uninduced for 4 days. The relative change of each target amplicon was determined in triplicate, and results from two separate experiments, with 18s rRNA as an internal control, were combined. On this log-scale graph, 1 indicates no change in the relative amount of RNA, while bars above 1 indicate an increase and bars below 1 indicate a decrease in the relative RNA amount. pre, pre-edited mitochondrial mRNA; ed, edited mitochondrial mRNA.

**Activities of KREPB9 and KREPB10 complexes.** The purified tagged editosomes contain the U addition (TUTase) and U removal (ExoUase) and RNA ligase (KREL1 and KREL2) activities indicating the presence of these proteins in the complexes and subcomplexes (see Fig. 4). The robust exoUase and ligase activities in the precleaved deletion activities reflect the preferential association of the deletion subcomplex with tagged KREPB9 and KREPB10. The TUTase activity is evident, since the products of precleaved insertion editing require U addition prior to ligase activity. The greater TUTase activity of the TEV eluate than of the CBE reflects the loss of the insertion subcomplex during the calmodulin column purification, as demonstrated by Western analysis. Thus, the insertion subcomplex is less stably associated with

tagged KREPB9 and KREPB10 proteins than is the deletion subcomplex.

The observation that tagged KREPB9 and KREPB10 complexes generate multiple 3' cleavage products with atypical mobilities (Fig. 4C and D) implies that the products have a 5' hydroxyl rather than a 5' phosphate (40, 46). These samples had a substantial proportion of ~10S complexes that typically lack endonucleolytic activity, and the amount of activity observed was less than is typically observed for the amount of material analyzed. A trivial explanation is that these products reflect contaminating noneditosome nuclease or 5' phosphatase. However, the enhanced generation of these products upon purification by CBE and confirmation by mass spectrometry that all three editing endonucleases were present in KREPB10 complexes suggest that cleavage products may be due to the complexes and reflect the association of KREPB9 or KREPB10 with the editosomes. These products may be due to postcleavage 5' phosphatase activity. Alternatively, they could result from cleavage 3' rather than 5' to the phosphate, although this is unprecedented among RNase IIIs. However, editosome-associated 3' phosphatase activity has been reported (33). Overall, these results suggest that KREPB9 and KREPB10 function may expand the endonuclease repertoire for recognition and cleavage of the hundreds of editing sites that are generally similar partial RNA duplex substrates but have distinct sequences and structures. KREPB9 or KREPB10 may also act in conjunction with MEAT1, given the reciprocal nature of the effect of their knockdowns on mRNA abundance *in vivo* (see below). Intriguingly, MEAT1 catalyzes U addition to substrates that lack a 5' phosphate (5), the apparent product that we observed with the tagged purified KREPB9 and KREPB10 editosomes.

**RNAi knockdown of KREPB9 and KREPB10.** The lack of growth inhibition upon RNAi knockdown of KREPB9 or KREPB10 implies that these proteins are not essential, at least in substantial amounts. However, since RNAi knockdown did not completely eliminate the target mRNAs, these proteins may yet be essential if they are required only in small amounts *in vivo*. The similarities among KREPB8, KREPB9, and KREPB10 also create the possibility that one of the related proteins could compensate for the loss of another. However, RNAi knockdown of KREPB8 causes a growth defect, indicating that neither KREPB9 nor KREPB10 can complement its loss (18). The effect of RNAi knockdown on mitochondrial mRNAs is particularly intriguing, because the loss of KREPB9 or KREPB10 decreases edited, preedited, and never-edited mRNAs, suggesting that these proteins function in a manner that goes beyond RNA editing *per se*, as discussed below.

**Effect on mitochondrial mRNA turnover.** Previous studies have indicated that editing can occur in conjunction with other mRNA processing events, such as polycistronic cleavage, polyadenylation, translation, and turnover (4, 5, 28, 32). Therefore, the decrease in mitochondrial mRNA abundance after KREPB9 or KREPB10 RNAi may reflect a relationship between editosome function and RNA turnover. Although some aspects of mitochondrial mRNA turnover have been identified, a cohesive understanding of the process by which both edited and never-edited transcripts are turned over has yet to emerge. Consequently, the relationships between editosome function and the processes involved in RNA stability and turnover remain only partially characterized. In *T. brucei*, a complex of DSS1 and SUV3 appears to be an ortholog of the yeast degradosome that is responsible for elim-

ination of transcripts, although the mechanism of transcript recognition is not clear (30, 31). Posttranscriptional 3' polyadenylation and polyuridylation status correlates with steady-state abundance of edited, preedited, and never-edited transcripts, albeit in a differential manner (16, 26, 42). Long (~200- to 300-nt) heterogeneous poly(A)/poly(U) tails added by KRET1/KPAP1/KPAF1/KPAF2 mark transcripts as competent for translation and correlate with their association with the small ribosomal subunit, while editosomes and their substrate RNAs associate with the large ribosomal subunit (4). Perhaps the most salient evidence showing an association between editosome function and RNA turnover is the RNAi knockdown of MEAT1, which resulted in a relative increase in all mitochondrial transcripts tested irrespective of editing (5). Because KREPB9 or KREPB10 knockdown has the opposite of the effect of MEAT1 knockdown, the functions of these editosome proteins may also impact the turnover of the mitochondrial transcripts. MEAT1 function has been hypothesized to result in degradation of misedited mRNAs, and thus the function of KREPB9 and KREPB10 would be expected to result in the accumulation of mitochondrial transcripts. These effects could be indirect as a result of these proteins affecting editosome recognition of mitochondrial transcripts, thus affecting their passage into the RNA turnover machinery.

**Endogenous KREPB9 and KREPB10.** The amounts of the KREPB9 and KREPB10 proteins in cells are uncertain although both seem to be low in abundance. The relatively lower frequency of peptides detected for the KREPB6, KREPB9, and KREPB10 proteins in numerous mass spectrometry experiments of editosome samples suggests that these proteins transiently interact with the editosomes (see Table S2 in the supplemental material). Neither KREPB9 nor KREPB10 was found in the mitochondrial proteome of procyclic trypanosomes (36), and they were not detected in hundreds of other mass spectrometric analyses performed in our lab. Both the KREPB9 and KREPB10 genes are expressed as mRNAs, as shown by qPCR (see Fig. 5), and their levels are not developmentally regulated (25). Peptides from endogenous KREPB10 were detected in TAP-tagged complexes isolated via MEAT1, chimeric KREN1-KREN2 endonuclease, and KREN1 from cells that repress KREPB8. Peptides from endogenous KREPB9 were not detected.

Because endogenous KREPB10 has been seen in only three types of TAP-tagged complexes, the unique qualities of these complexes provide clues to KREPB10 function. Its presence in MEAT1 editosomes suggests that the functional roles of these proteins may be associated, as is also suggested by the reciprocal effects on mitochondrial mRNA abundance resulting from their knockdown. Its presence in editosomes with tagged chimeric KREN2-KREN1 endonuclease supports a role associated with endonuclease function. The chimeric KREN2-KREN1 complexes also contain KREPB8 and KREX1, which are typically restricted to KREN1 editosomes, as well as KREPB7, which is typically restricted to KREN2 editosomes. Because these mass spectrometry results reflect the total population of isolated complexes, the composition of an individual complex is unclear. The simplest possibility is that KREPB7, KREPB8, and KREPB10 are mutually exclusive and occupy essentially the same site within an editosome. Alternatively, each complex contains more than one of these three proteins. Nevertheless, the altered structure of the chimeric endonuclease appears to promote the association of KREPB10 with the editosome, which presumably reflects interactions between the endo-

nuclease and KREPB10. The association of KREPB10 with endonuclease function is further supported by the discovery of endogenous KREPB10 in KREN1-TAP complexes isolated from cells in which KREPB8 is repressed. Peptides corresponding to KREPB10 were previously not observed in numerous experiments isolating KREN1 editosomes, either via TAP-tagged KREN1 or KREPB8 (10, 34). The presence of several KREPB10 peptides in 3 independent analyses of KREPB8 RNAi-KREN1TAP cells, however, indicates that repression of KREPB8 promotes association of KREPB10 with these editosomes. The sequence similarity between KREPB8 and KREPB10 suggests that KREPB10 has an interaction with KREN1 editosomes similar to that of KREPB8 but at a lower affinity. The observation of peptides for KREPB7 in KREN1-TAP complexes after KREPB8 repression similarly suggests that these proteins compete for a similar placement in the editosome. Taken together, these observations suggest that KREPB10 is involved in endonuclease function.

**Differential presence among species.** The differential presence of the KREPB9 and KREPB10 genes among kinetoplastid species is intriguing, considering the differences in which mitochondrial RNAs are edited and to what extent, as well as their different life cycles and associated metabolic changes between life cycle stages. Both genes are absent from species of *Leishmania*, which has about a third fewer uridine insertions and about half fewer uridine deletions than *T. brucei* (11). *T. vivax* lacks KREPB10, while *T. cruzi* retains both KREPB9 and KREPB10, even though *T. vivax* is more closely related to *T. brucei* than to *T. cruzi*. However, *T. vivax* is the most divergent member of the brucei clade, exhibiting differences in the maxicircle sequence and smaller minicircles than in *T. brucei* (7). Experimental analysis of RNA editing in *T. vivax* has not been reported. The absence of KREPB9 and KREPB10 within organisms that are capable of RNA editing (e.g., *Leishmania*) indicates that these proteins are not essential for editing.

**Functional roles of KREPB9 and KREPB10.** The data presented here characterize two newly identified editosome proteins, KREPB9 and KREPB10, that contain U1-like zinc finger and WGR motifs that have likely roles in molecular interactions. These interactions may be analogous to the spliceosomal U1C protein that promotes base pairing within the U1 snRNP that properly aligns RNAs during splicing and aids in spliceosome assembly. These motifs in KREPB9 and KREPB10 may have similar roles in affecting mRNA/gRNA interaction, alignment within the editosomes, association of its subcomplexes, and ultimately editing site recognition and cleavage. This may enable the endonucleases to recognize rare or unusual editing sites for cleavage. This implies a dynamic interaction of some proteins with the editosome as the numerous editing sites are processed. It might also provide for coordination of RNA editing with processing of the polycistronic transcripts from the maxicircle, since these processes are not temporally dissociated (28) and the decrease in mRNA abundance after KREPB9 or KREPB10 RNAi includes preedited and never-edited as well as edited transcripts. These results illustrate the integration of RNA editing with the other mitochondrial RNA processing activities.

**Roles of KREPB6 to -B10 in the dynamic editosome.** How the three distinct editosomes function *in vivo* to recognize and accurately edit the numerous different insertion and deletion editing sites is critical to understanding the overall process of RNA editing. One possibility is that the KREPB6 to B10 proteins function to modulate the activities and specificities of the endonucleases and



hence adapt them to the numerous diverse editing sites (i.e., substrates). KREPB6 to B10 associate directly or indirectly with the KREN1 to N3 endonucleases. KREPB6, KREPB7, and KREPB8 preferentially associate with the KREN3, KREN2, and KREN1 editosomes, respectively, and are involved in endonuclease function. KREPB6, KREPB7, and KREPB8 appear to be more restrictive in association with the endonucleases than KREPB9 and KREPB10. KREPB10 associates with all three types of editosomes. The low proportion of ~20S editosomes isolated via tagged KREPB9 prevents a similar assessment of how it associates with the editing endonucleases. Because the KREPB6 to B10 proteins all preferentially associate with the deletion subcomplex and they share significant sequence similarities, it seems likely that they occupy a similar physical location in an editosome. This hypothesis is supported by the observation that the repression of KREPB8 promoted the incorporation of either KREPB10 or KREPB7 into KREN1 editosomes. The three endonucleases also appear to share similar physical locations, based on the primary association with the insertion subcomplex. These data along with those indicating that the related KREPB6 to B8 are important to endonuclease activity and specificity suggest that multiprotein interactions affect and hence control the activities and specificities of the endonucleases.

Editosomes face the daunting tasks of recognizing numerous structurally similar but distinct mRNA/gRNA duplexes, aligning the mRNA strand with the endonuclease catalytic site, and cleaving it at the correct position prior to U addition or removal. In addition, the editosomes must subsequently progress from one editing site to another within a region specified by a single gRNA. The progression may be between two insertion sites or less frequently between insertion and deletion sites and even less frequently between two deletion sites. Furthermore, subsequent to the transfer of all the information in one gRNA, the mRNA must associate with the next gRNA to continue the process. The KREPB6 to B10 proteins may function as accessory factors to adapt the endonucleases to their substrates to accomplish these steps. The characteristics of these proteins, which may affect both the activities and specificities of catalytic and other proteins within a complex, illustrate how dynamic interactions among proteins in complexes may serve to adjust the overall functions of the complexes to multiple coordinated tasks and changing circumstances.

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