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RNA Editing in *Trypanosoma brucei* Requires Three Different Editosomes▽†

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***Trypanosoma brucei* has three distinct ~20S editosomes that catalyze RNA editing by the insertion and deletion of uridylates. Editosomes with the KREN1 or KREN2 RNase III type endonucleases specifically cleave deletion and insertion editing site substrates, respectively. We report here that editosomes with KREPB2, which also has an RNase III motif, specifically cleave cytochrome oxidase II (COII) pre-mRNA insertion editing site substrates in vitro. Conditional repression and mutation studies also show that KREPB2 is an editing endonuclease specifically required for COII mRNA editing in vivo. Furthermore, KREPB2 expression is essential for the growth and survival of bloodstream forms. Thus, editing in *T. brucei* requires at least three compositionally and functionally distinct ~20S editosomes, two of which distinguish between different insertion editing sites. This unexpected finding reveals an additional level of complexity in the RNA editing process and suggests a mechanism for how the selection of sites for editing in vivo is controlled.**

RNA editing recodes most of the mitochondrial mRNAs in trypanosomatids by the insertion and deletion of uridine nucleotides (U's) using information provided by guide RNAs (gRNAs) (58). RNA editing is developmentally regulated and is required for parasites that cycle between insect vector and mammalian host (54). Proteins that perform catalytic steps of RNA editing have been identified: endonucleases KREN1 or KREN2 cleave at deletion or insertion sites, respectively; terminal uridylyl transferase (TUTase) KRET2 adds U's at insertion sites; U specific exoribonuclease (exoUase) KREX1 removes U's at deletion sites; and ligases KREL1 or KREL2 rejoin mRNA fragments after U addition or removal (5, 13, 25, 30, 34, 55, 61). These catalytic steps are coordinated by the multiprotein editosomes that sediment at ~20S on glycerol gradients (9, 43). KREPB2 was identified as an editosome component by mass spectrometry, and found to contain an RNase III motif harboring amino acids that are critical for catalysis, a U1 Zn^{2+} finger, and double-stranded RNA binding. It has sequence similarity to KREN1 and KREN2, which also contain these three motifs (23, 41, 64). These data strongly suggest an RNA editing endonuclease role for KREPB2.

KREN1 and KREN2 were shown to be RNA editing endonucleases using both RNA interference (RNAi) and conditional knockout cell lines (5, 61). Both KREN1 and KREN2 are essential for the normal growth of PF and BF cells, and repression of their expression by either method led to dramatic growth defects or death, respectively. Such repression of KREN1 eliminated in vitro cleavage by ~20S editosomes of a deletion site in a synthetic substrate modeled on ATPase subunit 6 (A6) pre-mRNA but did not alter cleavage of an insertion site in a substrate derived from the same mRNA. These studies and other data have shown that KREN1 is an editing endonuclease with a preference for sites from which U's are deleted (29). Similarly, repression of KREN2 eliminated in vitro cleavage at insertion editing sites in substrates derived from A6 or cytochrome *b* (CYb) pre-mRNAs but did not alter cleavage at a deletion site in the A6 substrate. Repression of either KREN1 or KREN2 also resulted in a dramatic reduction in the relative amounts of several edited RNAs in vivo. Repression of KREPB2 by RNAi in procyclic forms, in contrast, had no significant growth phenotype, did not alter in vitro cleavage by editosomes of insertion or deletion sites in A6 substrates, and had little effect on edited RNAs in vivo except for a modest reduction of edited cytochrome oxidase II (COII) RNA (61).

A critical insight into editosome architecture has been the discovery that while ~20S editosomes have a common set of proteins, they are compositionally distinct, with KREN1, KREN2, and KREPB2 and a few associated proteins being mutually exclusive (39). Purified TAP-tagged KREN1, KREN2, or KREPB2 editosomes were also shown to be functionally distinct using in vitro enzymatic assays. While each type of ~20S editosome catalyzed terminal U transferase (TUTase), U-specific exonuclease (exoUase), and ligase activities, only KREN1 editosomes cleaved deletion sites, and only KREN2 editosomes cleaved insertion sites. Furthermore, editosome proteins KREPB8 and KREX1 were only found in KREN1 editosomes, KREPB7 was only found in KREN2 editosomes, and KREPB6 was only found in KREPB2 editosomes (A. K. Panigrahi et al., unpublished data). Complementary experiments with TAP-tagged KREPB6, KREPB7, and KREPB8 resulted in distinct editosomes with mutually exclu-

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sive KREPB2, KREN2, and KREN1, respectively (C. M. Zelaya Soares et al., unpublished data). The finding of KREPB2 in distinct editosomes with a composition that parallels that of two known editing endonucleases indicates that KREPB2 is likely to be an editing endonuclease.

We show here that KREPB2 is an RNA editing endonuclease that is essential for normal growth in bloodstream-form (BF) cells and hence rename it KREN3. Editosomes (~20S) and in vitro catalytic activities that cleave, add, or remove U's and ligate A6-derived RNA substrates persist after KREN3 repression. However, KREN3 repression eliminates COII RNA editing in vivo and similarly abolishes cleavage of a COII-derived RNA substrate in vitro. Mutations of key catalytic residues in the RNase III motif of KREN3 also eliminate COII cleavage in vitro and prevent the rescue of cells in which wild-type (WT) KREN3 is eliminated. Preferential cleavage of COII RNA by KREN3 editosomes indicates a novel RNA editing cleavage specificity.

MATERIALS AND METHODS

Plasmid constructs and transfections. Detailed descriptions of plasmid construction and transfections are in the supplemental material. Plasmids for making the RKO-KREN3 cell line were based on pLew13, pLew90, and pLew79 vectors (55, 63). Briefly, 416 bp of 5′ untranslated repeat (5′UTR) and 416 bp of 3′UTR from KREN3 flank the Neo^r marker of pLew13 to make pSKO-KREN3. The Neo^r marker is replaced with Hyg^r marker from pLew90 to make pDKO-KREN3. The KREN3 open reading frame is cloned into pLew79 to make pReg-KREN3 for tet-regulatable KREN3 expression. The RKO-KREN3 cell line was generated by a series of transfections to introduce the pSKO-KREN3, pReg-KREN3, and pDKO-KREN3 plasmids sequentially into BF (427 strain) cells. Proper integration of each plasmid was confirmed by PCR. In these cells, KREN3 expression is dependent upon the presence of tet.

TAP-tagged WT and mutated KREN3 genes were cloned into the pHD1344tub plasmid (53), creating the pWTN3-β, pE197V-β, pD204A-β, and pE271A-β plasmids. Transfections of the RKO-KREN3 cell line with pWTN3-β, pE197V-β, pD204A-β, or pE271A-β were performed to integrate constitutively expressed KREN3 alleles into the β-tubulin locus, making the RKO-WTN3-β, RKO-E197V-β, RKO-D204A-β, and RKO-E271A-β cell lines, respectively.

Growth curves. For BF growth curves, cells were grown to log phase and split to 2×10^5 cells/ml every 24 h in HMI-9 supplemented with 10% fetal bovine serum and appropriate antibiotics. Cell numbers were determined every 24 h by using a Coulter Counter. In BF cells containing the regulatable Reg-KREN3 allele, 1 μg of tet/ml maintained ectopic expression. To remove tet and repress the Reg-KREN3 allele, cells were centrifuged at 1,300 × *g* for 10 min, resuspended in media without tet, and then recentrifuged and resuspended a second time. Equal cell numbers were then grown in the absence or presence (by readdition) of tet.

Fractionation of cell lysates on glycerol gradients. Whole-cell lysates of BF RKO-KREN3 cells were prepared by resuspending ~1.3×10^9 cells in 900 μl of lysis buffer (10 mM Tris [pH 7.2], 10 mM $MgCl_2$, 100 mM KCl, 1 mM Pefabloc, 2 μg of leupeptin/ml, 1 μg of pepstatin/ml, 1 mM dithiothreitol [DTT]) and adding Triton X-100 to 1% with mixing by inversion for 15 min at 4°C. BF lysates were cleared by two centrifugation steps of 17,000 × g for 15 min at 4°C. BF lysates were loaded onto 10 to 30% glycerol gradients containing 20 mM HEPES (pH 7.9), 10 mM Mg, 50 mM KCl, and 1 mM EDTA and centrifuged at 38,000 rpm in a Beckman SW40 Ti rotor for 5 h at 4°C. Glycerol gradients were divided into 0.5-ml fractions from the top, flash frozen on liquid nitrogen, and stored at −80°C. For each sample within an experiment, equivalent cell numbers were lysed. Positive control ~20S samples from purified PF mitochondria (IsTaR 1.7a strain) or whole-cell lysates (427 strain) were generated as previously described (5, 57).

TAP-tag purifications. Editosome (~20S) complexes containing TAP-tag KREN1, KREN2, or KREN3 were purified from ~1.2×10^{10} cells using sequential immunoglobulin G (IgG) and calmodulin affinity chromatography with published PF cell lines as previously described (39, 46). This TAP protocol was scaled down and modified for purifications from BF cells as follows. Equivalent cell numbers (~2.1×10^8 BF cells) were harvested by centrifugation at 1,300 × *g* for 10 min and then resuspended in IPP150 buffer containing Complete protease inhibitors (Roche), 1 mM Pefabloc, 2 μg of leupeptin/ml, and 1 μg of pepstatin/ml to a final volume of 180 μl on ice. Cells were lysed by rotation at 4°C for 20 min after the addition of 20 μl of 10% Triton X-100. Cell lysates were centrifuged at 4°C at 9,000 × *g* for 15 min. Next, 3 μl of each cleared lysate was analyzed by Western blot, and 190 μl was then added to 10 μl of IgG-Sepharose beads that had been washed twice with 100 μl of IPP150 in a Handee Micro-Spin column (Promega). After 2 h of rotation at 4°C, columns were centrifuged at 3,000 × *g* for 1 min, followed by three washes of 250 μl of IPP150. IgG beads were subsequently washed once with 100 μl of TEVCB containing 1 mM DTT. TEV elution was performed in 30 μl of TEVCB containing 1 mM DTT and 1 μl of 10-U/μl AcTEV (Invitrogen) at 16°C for 2 h. Eluted protein was collected by centrifugation at 4°C at 3,000 × *g* for 1 min. Then, 10 μl of this TEV eluate was analyzed by Western blotting, and 7.5 μl was analyzed in COIIcisU1 cleavage assays.

Western blots. Glycerol gradient fractions (30 μl) were separated by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) gels, transferred to Immobilon-P membranes (Fisher), and probed using monoclonal antibodies against KREPA1, KREPA2, KREL1, and KREPA3 as previously described (40). To analyze TAP purified complexes, 7.5 μl of calmodulin eluate (PF) or 10 μl of TEV eluate (BF) was loaded and then blotted and probed as described above. Blots were stripped in 2% sodium dodecyl sulfate–62.5 mM Tris (pH 6.7)–100 mM 2-mercaptoethanol at 50°C for 30 min, then blocked in 1× phosphate-buffered saline with 0.05% Tween and 5% milk, and reprobed with a 1:500 dilution of α-calmodulin binding protein (CBP) rabbit polyclonal antibody (Millipore) and 1:2,000 goat α-rabbit IgG conjugated to HRP (Bio-Rad), followed by enhanced chemiluminescence (Pierce) and exposure to X-ray film.

In vitro enzymatic assays. Peak glycerol gradient fractions of ~20S complex were assayed, with either 5 or 15 μl for precleaved assays or 10 μl for endonuclease assays. Reactions were incubated at 28°C for 1 h (COII-derived substrates) or 3 h (A6-derived substrates). For all endonuclease and precleaved assays, RNAs were ethanol precipitated, resolved on 9% (COIIcisU1) or 11% (COIItransU1 and A6-derived substrates) polyacrylamide 7 M urea gels, and analyzed by using a PhosphorImager (Molecular Dynamics). The A6-derived substrate assays followed standard protocols described in detail elsewhere (5, 10, 28, 56). For A6-derived insertion endonuclease, cleavage of 70-nucleotide (nt) A6-eES1 pre-mRNA with gA6[14] gRNA was performed as described previously (5). For A6-derived deletion endonuclease, cleavage of 73-nt A6short/TAG.1 pre-mRNA with D34 gRNA was performed as described previously (5). For A6-derived precleaved editing, precleaved deletion and insertion editing were assayed as previously described using 5′-labeled U5 5′CL and U5 3′CL with gA6[14]PC-del and 5′-labeled 5′CL18 and 3′CL13pp with gPCA6-2A RNAs, respectively (26, 27). For COIIcisU1 insertion endonuclease, a DNA template for T7 transcription of COIIcisU1 was constructed by annealing COIIforUD and COIIrevWT PAGE-purified DNA oligonucleotides, followed by extension with *Taq* polymerase. For COIItransU1 insertion endonuclease, the oligonucleotides COIItransU1rev and gCOIItrans1rev were PAGE purified and then annealed to T7txnFOR to make DNA templates for Uhlenbeck T7 transcription of COIItransU1 and gCOIItrans1, respectively (35). After T7 transcription, COII-derived RNAs were PAGE purified, and 40 to 120 pmol of substrate RNA was radiolabeled by ligation to ^{32}P-labeled pCp (28, 56). Radiolabeled RNAs were subsequently purified to single nucleotide length on an 8% polyacrylamide 7 M urea gel. Each 30 μl of COIIcisU1 reaction contained ~2 pmol of substrate RNA (100,000 cpm), 22.5 mM HEPES (pH 7.9), 10 mM magnesium acetate, 0.5 mM DTT, 1 mM EDTA, 50 mM KCl, 2.5 mM $CaCl_2$, 8 U RNasin, and 0.5 μg of torula type VI RNA. Each 30 μl of COIItransU1 reaction contained ~2 pmol of substrate RNA (100,000 cpm) and 2.5 pmol of gCOIItrans1 in the same buffer.

Real-time PCR. Real-time PCR was performed as previously described (5). For the primers for COI, ND8pre, and ND8ed targets, see Table S1 in the supplemental material. Amplicons for these new primer sets were sequenced to confirm they amplified the specified target. The average of three cycle threshold (C_T) values for each target was used in calculations. Relative changes in target amplicons were determined by using the Pfaffl method, with PCR efficiencies calculated by linear regression using LinRegPCR (42, 45).

RESULTS

KREN3 repression inhibits growth. Repression of KREN3 expression in BF RKO-KREN3 cells inhibited growth (Fig. 1). The RKO-KREN3 cell line was created by eliminating both endogenous KREN3 alleles by homologous recombination af-

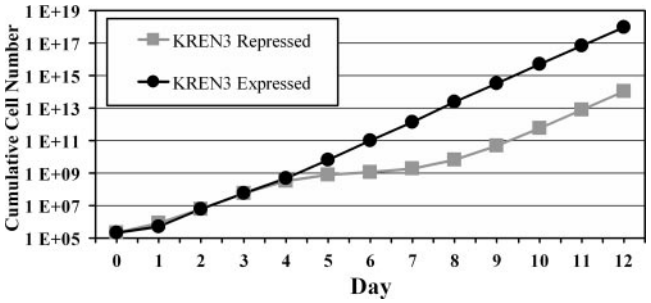

FIG. 1. KREN3 repression inhibits growth. Growth of the BF RKO-KREN3 cells in the presence of tet (●), which allows KREN3 expression, or without tet (▨), which represses KREN3 expression. Growth eventually resumes due to the loss of tet regulation and reexpression of KREN3.

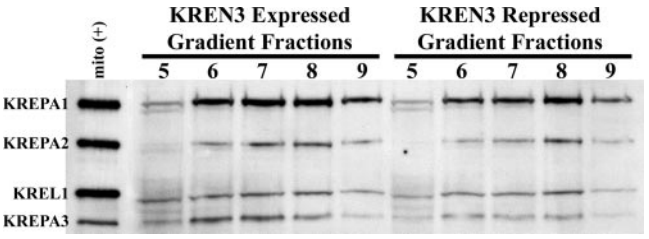

FIG. 2. KREN3 repression does not alter editosome sedimentation. Western analysis of lysates of BF RKO-KREN3 cells that were grown with (KREN3 expressed) or without (KREN3 repressed) tet for 3 days. Fractions from glycerol gradients were probed with a mixture of monoclonal antibodies that are specific for KREPA1, KREPA2, KREL1, and KREPA3 editosome proteins. Fractions 6 to 8 are the ~20S peak of the gradient. A ~20S fraction from purified PF mitochondria, mito(+), is used as a positive control.

ter introduction of an inducible Reg-KREN3 allele into the rDNA intergenic locus. Allele elimination and the intended genomic integration of the introduced KREN3 allele were confirmed by PCR analysis at the 5′ and 3′ integration boundaries (data not shown). Removal of tetracycline (tet), which is required for expression of the Reg-KREN3 allele, resulted in decreased cell growth after 3 days (Fig. 1). The subsequent resumption of cell growth was accompanied by reexpression of KREN3 mRNA at prerepression levels as shown by real-time PCR analysis at 12 days (data not shown), indicating loss of tet regulation. The loss of cell growth upon loss of KREN3 expression and resumption of growth upon reexpression indicates that KREN3 is an essential gene in *T. brucei*.

In vitro editing activities are retained after loss of KREN3. Editosomes that sediment at ~20S in glycerol gradients were present after 3 days of KREN3 repression (Fig. 2), a time point at which KREN3 mRNA is significantly repressed (see Fig. 4). Although editosomes were slightly decreased in amount compared to when KREN3 is expressed, they contained normal proportions of the four proteins for which we have monoclonal antibodies. The peak ~20S editosome fractions catalyzed cleavage of insertion and deletion site substrates, as well as the subsequent U addition, U deletion, and RNA ligation activities in standard in vitro assays (Fig. 3). These fractions, from cells in which KREN3 was expressed or repressed for 3 days, cleaved insertion editing site substrate RNAs derived from ATPase subunit 6 (A6) editing site 2 (Fig. 3A) and deletion editing site substrate RNAs derived from A6 editing site 1 (Fig. 3B). These fractions also catalyzed TUTase and ligase or exoUase and ligase activities with precleaved insertion (Fig. 3C) or precleaved deletion (Fig. 3D) substrates. Thus, the ~20S editosome from cells in which KREN3 expression was repressed retained the general catalytic activities characteristic of RNA editing.

KREN3 repression results in a loss of edited COII RNA in vivo. Real-time PCR analysis revealed that KREN3 repression preferentially and dramatically reduced COII editing in vivo (Fig. 4). Total RNA was isolated from cells in which KREN3 was expressed or repressed for 3 days, treated with DNase I, reverse transcribed into cDNA, and then analyzed by real-time PCR. Relative amounts of each target RNA were calculated by using β-tubulin and 18S rRNA internal controls. The average standard deviation of measured C_T values was 0.017, and the standard deviation of three independent experiments of relative KREN3 levels was 0.013 (not shown). As expected, the amount of KREN3 mRNA was dramatically reduced, whereas KREN1 and KREN2 levels remained unchanged. Maxicircle

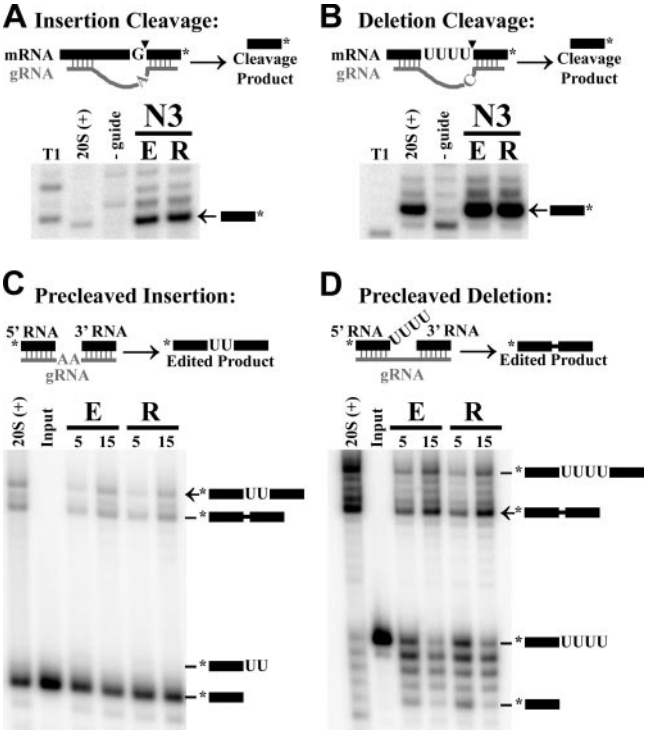

FIG. 3. KREN3 repression does not alter in vitro editing activities on an A6-derived substrate. The ~20S glycerol gradient fractions from BF RKO-KREN3 cells grown for 3 days in the presence (E, expressed) or absence (R, repressed) of tet were incubated with A6-derived RNA substrates to assay endonucleolytic cleavage of insertion (A) or deletion (B) and precleaved insertion (C) or deletion (D) editing activities. The RNA substrates and products are shown schematically with the asterisk indicating radiolabel and the wedge indicating the cleavage site. The cleavage products in panels A and B (arrows) are due to RNA editing endonuclease activity, as indicated by the requirement for gRNA (-guide). Lanes with radiolabeled substrate (input) or positive control ~20S glycerol gradient fractions from PF lysates [20S(+)] are indicated. The amounts of fractions used in panels C and D are indicated in microliters above each lane. T1-digested substrate RNA is used as a marker to localize the cleavage site.

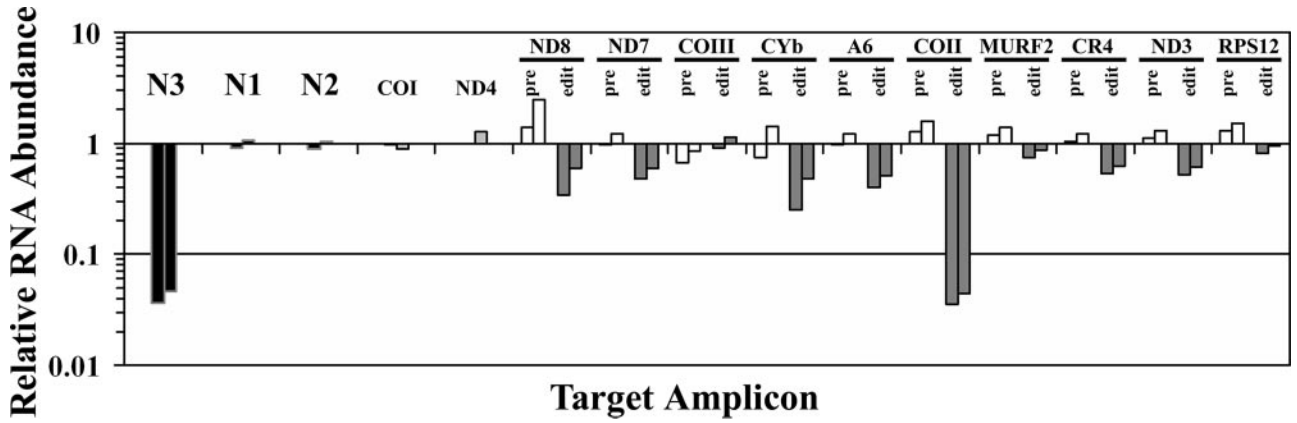

FIG. 4. KREN3 repression inhibits COII editing in vivo. Real-time PCR analysis comparing total RNA from BF RKO-KREN3 cells in which KREN3 expression was repressed for 3 days with RNA from cells in which KREN3 was expressed. The relative change of each target amplicon is determined in triplicate with either β-tubulin (left bar) or 18S rRNA (right bar) as an internal control. Preedited mRNA is indicated by white bars, edited mRNA is indicated by dark gray bars, mRNA for RNase III-motif-containing proteins is indicated by black bars, and mRNA that is never edited is indicated by light gray bars. On this log-scale graph, 1 indicates no change in relative amount of RNA, while bars above 1 indicate an increase and bars below 1 indicate a decrease in relative RNA amount.

transcripts that do not get edited, such as COI and ND4, were also not altered by KREN3 repression. The amount of edited COII, however, was severely reduced (by 96%) upon the loss of KREN3 expression. Other edited RNAs, including ND8, ND7, CYb, A6, CR4, and ND3, were reduced between 40 and 75%, whereas edited RNAs for COIII, MURF2, and RPS12 were essentially unaltered. The amounts of preedited RNA were unchanged or slightly increased after KREN3 repression.

KREN3 repression inhibits endonucleolytic cleavage of COII in vitro. The dramatic decrease in edited COII levels in vivo after KREN3 repression impelled us to investigate in vitro cleavage of RNA substrates derived from COII pre-mRNA. RNA substrates derived from COII pre-mRNA undergo pre-cleaved editing in vitro (18, 19) and are cleaved by mitochondrial extracts (D. E. Golden and S. L. Hajduk, unpublished data). We designed the 81-nt COIIcisU1 substrate based on

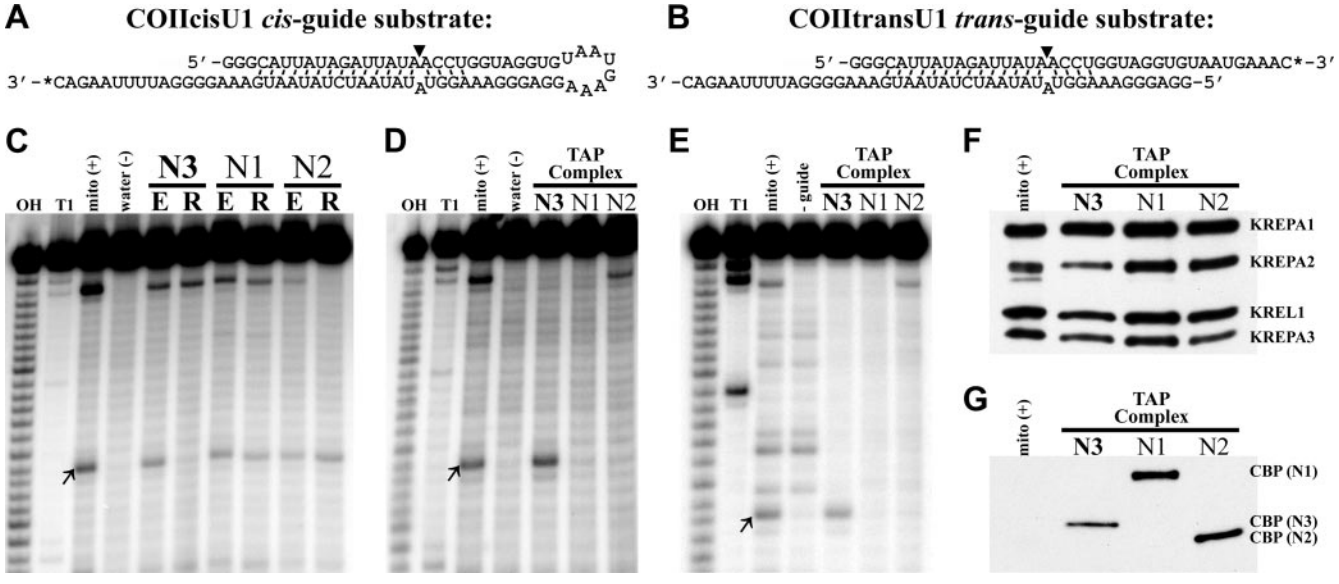

FIG. 5. KREN3 repression eliminates COII endonuclease activity in vitro. COII-derived *cis* (A) and *trans* (B) substrate RNAs with the radiolabel and cleavage site indicated by an asterisk and wedge, respectively. Cleavage assays with *cis* substrate RNA COIIcisU1 using ~20S glycerol gradient fractions of lysates from BF RKO-KREN3 (N3), BF RKO-KREN1 (N1), or BF RKO-KREN2 (N2) cells grown for 3 days in the presence (E, expressed) or absence (R, repressed) of tet (C) or purified PF TAP-tagged editosomes (D). The cleavage product (arrow) is mapped relative to alkaline hydrolysis (OH) and T1-digested substrate RNA (T1) ladders. "mito (+)" is an ~20S fraction from purified PF mitochondria used as a positive control, and "water (−)" is used as a negative control. Note that cleavage only occurs in editosomes that contain KREN3. Western analysis of the TAP-tagged editosomes with monoclonal antibodies against KREPA1, KREPA2, KREL1, and KREPA3 editosome proteins (F) and with antibody against the CBP tag upon reprobing the stripped blot (G). Cleavage assays with the *trans* substrate RNA using the TAP-tagged editosomes (E). Loss of cleavage upon omission of gRNA (-guide) shows that cleavage is due to RNA editing endonuclease activity.

COII mRNA, and like COII mRNA it has the gRNA sequence in *cis* (Fig. 5A). Unlike COII pre-mRNA, this substrate has only one editing site for a single U insertion, and a portion of the intervening RNA between the editing site and the guiding sequence was removed. COIIcisU1 was transcribed in vitro from a DNA template by T7 polymerase, radiolabeled, gel purified, and used in cleavage assays.

Cleavage of COIIcisU1 substrate at the COII editing site required KREN3 (Fig. 5). A 64-nt cleavage product that corresponds to editing site 1 of COII was generated by ~20S editosome fractions from PF mitochondria and BF cells that expressed KREN1, KREN2, or KREN3 and from cells in which expression of KREN1 or KREN2 was repressed (Fig. 5C). In contrast, fractions from BF cells in which expression of KREN3 was repressed and the negative control (water) did not generate the cleavage product. Thus, KREN3 editosomes appear to cleave RNA substrates derived from COII pre-mRNA.

Direct analysis with TAP-tag-purified KREN3 editosomes from PFs showed that they cleaved COIIcisU1 RNA substrate in vitro, as did ~20S editosomes from isolated mitochondria, while TAP-tag-purified PF KREN1 and KREN2 editosomes did not (Fig. 5D). Published results have shown that TAP-tag-purified PF KREN3 complexes cleave neither A6-dervied insertion nor deletion substrates, whereas PF KREN1 and KREN2 editosomes cleave deletion and insertion sites, respectively (39). To simultaneously test both the dependence for gRNA on COII-derived cleavage and whether KREN3 specificity is due to this substrate's *cis*-guided nature, the COIItransU1 substrate was created (Fig. 5B). A 21-nt cleavage product was generated by PF ~20S mitochondrial fractions, and this cleavage required gCOIItrans1 gRNA (Fig. 5E). TAP-tagged PF KREN3 editosomes also generated this cleavage product, but TAP-tagged PF KREN1 or KREN2 editosomes did not. Thus, KREN3 is required for cleavage of both *trans*- and *cis*-guided COII-derived substrates in vitro. An additional cleavage product that requires KREN2 was observed using either BF lysates or PF TAP-purified editosomes, and it mapped 3 nt from the 5′ end for both COIIcisU1 and COII transU1 substrates (Fig. 5C, D, and E). Western analysis shows that the TAP-tagged editosomes all contain similar amounts of KREPA1, KREPA2, KREL1, and KREPA3, as well as the TAP-tagged protein (Fig. 5F and G).

RNase III mutations eliminate COII endonuclease activity. Conserved amino acids in the RNase III motif of KREN3 are critical for function in vivo and in vitro (Fig. 6). Three different KREN3 alleles that contain single amino acid substitutions in the RNase III motif (E197V, D204A, or E271A) and a WT KREN3 control were tested for the ability to rescue the growth phenotype of BF RKO-KREN3 cells (see Fig. S1 in the supplemental material). These alleles were TAP tagged to facilitate monitoring of expression and subsequent purification of editosomes and introduced into the β-tubulin locus of BF RKO-KREN3 cells to create cell lines (RKO-E197V-β, RKO-D204A-β, RKO-E271A-β, and RKO-WTN3-β, respectively) that constitutively expressed these KREN3 alleles. In these cell lines, the KREN3 allele in the β-tubulin locus is exclusively expressed when the WT Reg-KREN3 (from the rRNA intergenic locus) is repressed by the removal of tet, as shown by real-time PCR (data not shown). Each of these cell lines grew continuously in the presence of tet at similar rates (Fig. 6A).

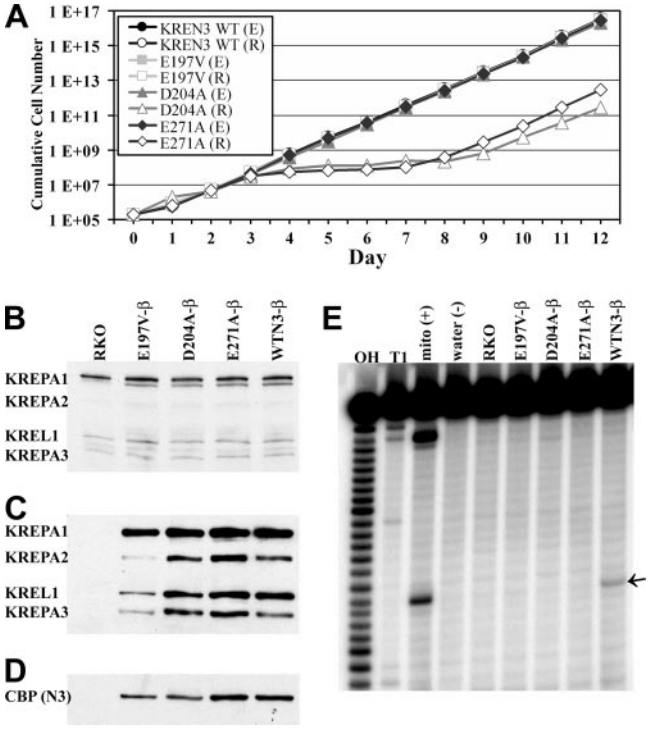

FIG. 6. Key residues in the RNase III motif of KREN3 are required for growth and COII-derived substrate RNA cleavage in vitro. (A) Growth of RKO-WTN3-β, RKO-E197V-β, RKO-D204A-β, and RKO-E271A-β cell lines with the Reg-KREN3 allele expressed (E) or repressed (R), as indicated in the inset. The D204A and E271A alleles fail to rescue for loss of KREN3, while the E197V and WT allele do rescue. (B) Western analysis of whole-cell lysates from BF RKO-KREN3 (RKO), RKO-WTN3-β, RKO-E197V-β, RKO-D204A-β, and RKO-E271A-β cells probed with monoclonal antibodies against KREPA1, KREPA2, KREL1, and KREPA3. This shows that an equivalent amount of material was used in each affinity purification. (C) Western of TEV eluates after IgG affinity purification from lysates shown in panel B using monoclonal antibodies against KREPA1, KREPA2, KREL1, and KREPA3. (D) The blot from panel C stripped and reprobed with antibody against CBP tag on KREN3 proteins. (E) COII-derived substrate RNA cleavage by TEV eluates examined in panels C and D. The cleavage product (arrow) is localized relative to alkaline hydrolysis (OH) and T1-digested substrate RNA (T1). Note that editosomes with WT KREN3 cleave COII-derived substrate RNA, while those with mutant KREN3 do not. "Mito (+)" is a positive control lane of ~20S fraction from purified PF mitochondria, while omission of editosomes [water (−)] or tagged editosomes (RKO) are negative controls.

Repression the Reg-KREN3 allele by removal of tet resulted in a severe growth inhibition in both RKO-D204A-β and RKO-E271A-β cell lines after 3 days. Growth inhibition at day 3 was more acute in both RKO-D204A-β and RKO-E271A-β than in the parental BF RKO-KREN3 line. However, there was no growth inhibition upon Reg-KREN3 repression in either the RKO-E197V-β or the RKO-WTN3-β cell lines. The point mutation in the RKO-E197V-β cell line was confirmed by sequencing PCR products that were amplified from genomic DNA (data not shown). The ability of both WT and E197V KREN3 to rescue growth upon KREN3 repression indicates that their protein products retained function, while the failure of the D204A and E271A alleles to compensate for

the loss of KREN3 indicates that the RNase III motif is essential for KREN3 function and in turn cell viability.

Mutations in the RNase III motif of KREN3 ablated COII cisU1 cleavage by purified editosomes in vitro (Fig. 6B to E). Western analysis revealed that editosome proteins were similar in abundance in total cell lysates of equivalent numbers of RKO-KREN3, RKO-E197V-β, RKO-D204A-β, RKO-E271A-β, and RKO-WTN3-β cells (Fig. 6B). IgG-Sepharose affinity chromatography, followed by TEV protease elution of TAP-tagged editosomes, resulted in similar relative proportions of editosome proteins (Fig. 6C). Yields of the tagged editosomes were similar, with somewhat less recovered from RKO-E197V-β cells. Stripping and reprobing the blot with anti-calmodulin binding peptide revealed similar abundance of the tagged mutated or WT KREN3 proteins in each sample (Fig. 6D). Thus, the tagged proteins were integrated into the editosomes. Editosomes were not recovered from the parental BF RKO-KREN3 cells by this method since they did not contain a TAP-tag and thus serve as a negative control. While the purified WT KREN3 editosomes cleaved COIIcisU1 substrate, none of the purified editosomes that contain mutant KREN3 cleaved this substrate (Fig. 6E). Thus, while the E197V mutation was not sufficient to prevent KREN3 function in vivo, it was sufficient to prevent cleavage of the COIIcisU1 substrate in vitro. These results indicate that each of three mutated amino acids in the RNase III motif of KREN3 function in KREN3 activity in vitro.

DISCUSSION

We show here that KREPB2 is an RNA editing endoribonuclease, one that preferentially functions in the cleavage of COII mRNA in vivo and in vitro. KREPB2 is thus renamed KREN3 by convention to indicate its endonucleolytic function (58), albeit out of numerical order by molecular size due to the earlier naming of KREN2 (5). KREN3 is essential in *T. brucei*, as shown by the inhibition of parasite growth following its repression and subsequent recovery upon its reexpression. The lack of growth inhibition upon RNAi repression of KREN3 in PF that was previously reported (61) probably reflects the incomplete knockdown of KREN3 expression. Hence, RNA editing is normally essential in bloodstream and insect vector forms of *T. brucei*, as shown by growth inhibition after repression of KREL1, KREPB5, KREN2, KREN1, KRET2, or now KREN3 (5, 11, 55, 61, 62). Repression of KREN3 expression resulted in selective inhibition of COII editing in vivo and the loss of in vitro cleavage of a COII-derived RNA substrate by ~20S editosomes from these cells. In addition, purified TAP-tagged KREN3 editosomes, but not KREN1 or KREN2 editosomes, cleaved COII-derived RNA substrates in vitro. Mutations in the RNase III motif of KREN3 ablated this in vitro cleavage, showing that KREN3 is an editing endonuclease. Expression of an allele with either D204A or E271A mutations, unlike the WT KREN3 allele, failed to rescue cells from growth inhibition after KREN3 repression, thus indicating that the catalytic activity of KREN3 is essential in vivo. Repression of KREN3 reduced the total amount of ~20S editosomes implying that KREN3 editosomes were specifically lost while KREN1 and KREN2 editosomes remained. Similar results were observed upon repression of KREN1 or KREN2 (5, 61). Overall, KREN3 is the endonucleolytic component of one of three major distinct types of ~20S editosomes, each of which also has TUTase, exoUase, and ligase activities. Each of these ~20S editosomes preferentially cleaves and hence edits different mRNAs and/or editing sites.

The most striking effect on editing in vivo after KREN3 repression is the dramatic reduction of edited COII RNA, which mirrors the loss of KREN3 mRNA (Fig. 4). In contrast, repression of either KREN1 or KREN2 results in large reductions in edited ND7, COIII, A6, MURF2, ND3, and RPS12 RNAs but only a relatively small decrease in edited COII RNA (5, 61). This suggests that editing of all three sites of COII in vivo is catalyzed by KREN3 editosomes. KREN3 repression results in a fewfold relative reduction of some other edited RNAs (ND8, ND7, CYb, A6, CR4, and ND3) but has essentially no effect on the relative levels of others, including those for edited (COIII, MURF2, and RPS12), never-edited (COI and ND4), or editosome protein (KREN1 and KREN2) mRNAs. The decrease in edited ND8, ND7, CYb, A6, CR4, and ND3 RNA levels suggests the possibility that KREN3 editosomes may cleave, and hence edit, sites in these RNAs, as well as in COII RNA. Alternatively, these relatively small decreases, which resemble the effect on edited COII RNA after KREN1 or KREN2 repression, imply that they may be secondary effects, although RNA was isolated before inhibition of cell growth was evident.

The lethality for BF *T. brucei* upon KREN3 repression is puzzling since normal BFs, which rely on glycolysis, have less edited COII mRNA than PFs (2, 14, 15) and lack cytochrome *c* oxidase (complex IV) activity (16, 44, 51, 52). Indeed, BF cytochrome *c* oxidase subunit 6 null mutants are viable (60). One possibility is that BFs require the COII protein either for complex IV function or for an unknown function. Such an unknown function may be analogous to that of ATP synthase complex, since it functions in reverse in BFs compared to PFs (53), and its α subunit has been reported to actively import tRNAs into *Leishmania* mitochondria (20). Alternatively, the lethality may reflect loss of editing of other mRNAs, as suggested above, or KREN3 may have an additional unknown function such as cleavage of polycistronic maxicircle or minicircle transcripts (21).

The basis for editing site recognition by KREN3 editosomes is uncertain. The COII guiding sequence need not be in the 3′UTR, at least in vitro (Fig. 5), indicating that recognition by KREN3 does not depend on structural features resulting from a *cis* guiding sequence. Nevertheless, the tertiary structure of the interacting gRNA/mRNAs may be critical to editosome endonuclease specificity (32, 33). Indeed, cleavage site specificity by other RNase IIIs is influenced by tertiary structure, particularly internal loops (36, 47, 66). Some aspect of COII mRNA sequence may play a role in its recognition by KREN3 editosomes, since inhibitory sequences (antideterminants) have been identified for *E. coli* RNase III (66). However, sequence alone seems unlikely to be sufficient for the differential recognition by KREN1, KREN2, and KREN3 editosomes because many hundreds of different sequences exist at editing sites. General characteristics that distinguish insertion and deletion editing sites, such as unpaired A's and G's in gRNA at insertion sites and unpaired U's in mRNA at deletion sites, might play a role in recognition by editosomes (4). Con-

version of an insertion site into a deletion site is consistent with this notion (8). However, KREN3 and KREN2 editosomes both cleave insertion editing sites and thus must have another basis for discriminating between their respective editing sites.

KREN3 recognition and cleavage of its substrates likely entails interactions with other editosome proteins. Editing endonuclease activity is typically associated with ~20S editosomes while TUTase, ExoUase, and RNA ligase activities are seen in 5-10S subcomplexes and in recombinant proteins (1, 13, 29, 30, 38, 40, 49). Although in vitro cleavage has been observed with recombinant KREN1, 24% cleavage activity remained after deletion of the entire RNase III motif in these studies (29). Catalysis by RNase III endonucleases invariably uses two interacting RNase III domains (3, 37). Bacterial and yeast RNase IIIs form homodimers, while the two RNase III domains of mammalian dicer form an intramolecular dimer. Only one of the editing endonucleases appears to be present in each ~20S editosome (J. Carnes et al., unpublished data), leading us to hypothesize that KREN1, KREN2, and KREN3 form heterodimers with KREPB4 and/or KREPB5. Both KREPB4 and KREPB5 have RNase III motifs that lack key residues, implying that they are catalytically inactive, and both play critical roles in ~20S editosome stability (1, 62). Their RNase III domains may interact with that of KREN1, KREN2, and KREN3 to create a catalytic site that only cleaves mRNA in the mRNA/gRNA duplex. While RNase IIIs typically cleave both strands of an RNA duplex, cleavage of one strand has been reported, and yeast Rnt1p can catalyze guide-directed cleavage of a single strand (6, 7, 31, 48, 50). Having two different proteins, i.e., KREPB4 and KREPB5, may expand the repertoire of editing sites that the editing endonucleases can recognize and cleave.

The exclusive presence of KREPB6 with KREN3, KREPB7 with KREN2, and KREPB8 with KREN1 in purified ~20S editosomes implies that these proteins contribute to the specific function of each of these editosomes (39; Panigrahi et al., unpublished; Zelaya Soares et al., unpublished). The function of KREPB6, KREPB7, and KREPB8 may be associated with the preferential recognition of different editing sites by the different types of ~20S editosomes and may be analogous to the role DGCR8 plays in specifying cleavage site by Drosha (12, 22, 24). Indeed, editosome endonuclease activity may function in the context of heterotrimers. Six compositionally different heterotrimeric endonuclease subcomplexes may function to recognize various editing sites if they differentially use KREPB4 or KREPB5 with KREN1/KREPB8, KREN2/KREPB7, or KREN3/KREPB6.

The loss of function in vivo and in vitro resulting from mutations in the RNase III motif of KREN3 indicates its role as an endonuclease. Its conserved amino acids D204 and E271 correspond to D44 and E110 of *Aquifex aeolicus* RNase III, residues that coordinate the catalytic divalent cation in the crystal structure and are essential for catalysis (3, 17, 65). Loss of cleavage activity in vitro by D204A or E271A mutated KREN3 indicates that KREN3 itself catalyzes the endonucleolytic cleavage when in editosomes. Presumably, its mechanism of cleavage is similar to that of *Aquifex aeolicus* RNase III. The inability of KREN3 with these mutations to rescue the viability following loss of WT KREN3 indicates that its activity, and hence KREN3 editosomes, are essential. The more rapid onset of growth inhibition of these mutants compared to repression of KREN3 expression alone may reflect the presence of mutant nonfunctional KREN3 editosomes. Nonfunctional editosomes might bind substrate RNA, thus sequestering it and inhibiting overall editing.

The inability of KREN3 editosomes with the E197V mutation to cleave COII substrate RNA in vitro and yet rescue cells from inactivation of KREN3 expression in vivo may reflect a role for E197 in substrate and/or protein interaction. Mutation of the equivalent residue in *Escherichia coli* RNase III reduced activity in vitro and increased the K_m (3, 59, 65). Analysis of the crystal structure of *A. aeolicus* suggests that its corresponding residue is involved in RNase III subunit dimerization (17). The E197V mutation might cause reduced substrate binding by KREN3 editosomes so that cleavage is not detectable in vitro, but decreased reaction kinetics may permit sufficient product to accumulate in vivo. After KREN1 or KREN2 repression, the expression of alleles with the mutation equivalent to KREN3 E197V failed to rescue cells and resulted in impaired in vitro cleavage activity by ~20S editosomes (5, 61). The different effects of the E197V mutation in KREN3 versus KREN1 and KREN2 may reflect differences in how these three endonucleases interact with other editosome proteins and substrates and hence mirror different specificities in their editing site recognition and cleavage.

The results reported here expand the functional distinctions among the KREN1, KREN2, and KREN3 ~20S editosomes. These editosomes preferentially cleave distinct editing sites, revealing that different insertion editing sites can be distinguished as unique substrates. To date, each in vitro cleavage substrate tested has been cleaved by only one of the three identified editing endonucleases, suggesting that most, if not all, editing sites will be similarly segregated. The requirement of three compositionally distinct ~20S editosomes with three distinct endonucleolytic specificities implies additional complexity in RNA editing that may form the basis of recognizing diverse editing sites and perhaps regulating editing.

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