Identification by Random Mutagenesis of Functional Domains in KREPB5 That Differentially Affect RNA Editing between Life Cycle Stages of *Trypanosoma brucei*

Suzanne M. McDermott, Jason Carnes, Kenneth Stuart

Center for Infectious Disease Research, Seattle, Washington, USA

KREPB5 is an essential component of ~20S editosomes in *Trypanosoma brucei* which contains a degenerate, noncatalytic RNase III domain. To explore the function of this protein, we used a novel approach to make and screen numerous conditional null *T. brucei* bloodstream form cell lines that express randomly mutagenized KREPB5 alleles. We identified nine single amino acid substitutions that could not complement the conditional loss of wild-type KREPB5. Seven of these were within the RNase III domain, and two were in the C-terminal region that has no homology to known motifs. Exclusive expression of these mutated KREPB5 alleles in the absence of wild-type allele expression resulted in growth inhibition, the loss of ~20S editosomes, and inhibition of RNA editing in BF cells. Eight of these mutations were lethal in bloodstream form parasites but not in procyclic-form parasites, showing that multiple domains function in a life cycle-dependent manner. Amino acid changes at a substantial number of positions, including up to 7 per allele, allowed complementation and thus did not block KREPB5 function. Hence, the degenerate RNase III domain and a newly identified domain are critical for KREPB5 function and have differential effects between the life cycle stages of *T. brucei* that differentially edit mRNAs.

The kinetoplastid parasite *Trypanosoma brucei* is the etiologic agent of human African trypanosomiasis, which is transmitted by the tsetse fly and is a health threat to millions of people in sub-Saharan Africa. *T. brucei* is a deeply divergent eukaryote, the study of which advanced the understanding of many fundamental biological processes and eukaryotic evolution. Indeed, a number of these processes, such as *trans* splicing, polycistronic transcription, antigenic variation, glycosylphosphatidylinositol anchoring, and mitochondrial RNA editing, were first described in trypanosomes and provided novel paradigms for eukaryotic biology (1–11). Kinetoplastids are named for their distinctive mitochondrial DNA network, known as the kinetoplast DNA (kDNA), within their single mitochondrion. The kDNA in *T. brucei* is comprised of ~50 identical maxicircles and thousands of heterogeneous minicircles (12, 13). The ~22-kb maxicircles encode two rRNAs and mRNAs for 18 mitochondrial proteins, 12 of which undergo posttranscriptional RNA editing to generate translatable open reading frames (ORFs). RNA editing involves the precise insertion and deletion of uridylylates (Us) at hundreds and tens of editing sites (ESs), respectively. The minicircles encode numerous diverse ~60-nucleotide guide RNAs (gRNAs) which specify edited sequences (14–16). Editing progresses generally, but not precisely, 3' to 5' with respect to the mRNA, each gRNA specifies the editing of numerous ESs, and multiple gRNAs are required for complete editing of most transcripts. *T. brucei* undergoes stage-specific adaptations in the bloodstream of the mammalian host and in the tsetse fly, particularly in mitochondrial function (17, 18), and a number of maxicircle transcripts are differentially edited between bloodstream form (BF) and insect, procyclic-form (PF) cells. The molecular basis of this developmental control is unknown, but differential gRNA utilization has been suggested to play a role (19, 20).

RNA editing occurs by rounds of coordinated catalytic steps: cleavage of the mRNA by endonuclease and addition of Us by 3' terminal uridylyltransferase (TUTase) or removal of Us by U-specific 3' exonuclease (exoUase) at insertion and deletion ESs, re-

spectively, followed by rejoining of the mRNA fragments by RNA ligase. The enzymes that catalyze RNA editing are in ~20S multi-protein editosome complexes that also contain proteins that have no known catalytic functions (14, 21–31). Three similar versions of these ~20S complexes exist, and all of them have a common set of 12 proteins, but each contains a different RNase III endonuclease and specific partner protein (25–29, 32). These distinct ~20S editosomes differ in their ES cleavage specificity (27–29). One complex contains the KREN1/KREPB8 protein pair and the KREX1 3' exonuclease and cleaves deletion ESs. The other two complexes contain the KREN2/KREPB7 or KREN3/KREPB6 protein pairs and cleave insertion sites, albeit with different preferences.

KREPB5 is 1 of 12 proteins common to all ~20S editosomes and is essential in both BF and PF cells, where it is required for RNA editing *in vivo* (33, 34). Knockdown of KREPB5 expression results in the loss of intact editosomes and editosome proteins in BFs, while in PFs there is a partial retention of ~20S complexes and a shift of editosome components to smaller S values (33, 34). Prediction of the protein sequence and structural motifs revealed that KREPB5 and a second editosome protein, KREPB4, each contain a U1-like zinc finger (ZnF) motif (which is degenerate in

Received 11 August 2015 Returned for modification 3 September 2015
Accepted 8 September 2015

Accepted manuscript posted online 14 September 2015

Citation McDermott SM, Carnes J, Stuart K. 2015. Identification by random mutagenesis of functional domains in KREPB5 that differentially affect RNA editing between life cycle stages of *Trypanosoma brucei*. *Mol Cell Biol* 35:3945–3961. doi:10.1128/MCB.00790-15.

Address correspondence to Kenneth Stuart, ken.stuart@cidresearch.org.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/MCB.00790-15>.

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KREPB5), a degenerate RNase III domain, and a PUF (Pumilio and fem-3 binding factor) motif (35, 36). Previous studies have sought to analyze the precise role of KREPB5 by making targeted mutations in residues predicted to be crucial for the function of these domains. A total of 10 residues were analyzed, and three RNase III domain residues (R77, F114, and G121) that are essential for KREPB5 function in BF parasites were identified (34, 35). All characterized RNase III domains function as dimers to cleave both strands of double-stranded RNA (dsRNA). Replacement of the highly conserved glycine at residue 121 with arginine is thought to prevent KREPB5 function by creating steric interference in an α helix important for RNase III domain dimerization. Exclusive expression of the G121R mutant KREPB5 resulted in severe inhibition of growth, inhibition of RNA editing, and a loss of editosome components and complexes (34, 35). A second substitution, F114S, was also predicted to result in conformational changes that would affect RNase III dimerization and/or the catalytic efficiency of an opposing RNase III domain. Exclusive expression of F114S mutant KREPB5 resulted in a moderate growth defect, underlining the importance of the KREPB5 RNase III domain *in vivo* (35). In contrast, mutation of KREPB5 residues that correspond to conserved catalytic RNase III residues has no effect on editing or *in vitro* cleavage of ESs (35). Previous studies have shown that the editing endonucleases do not form homodimeric RNase III domains (26), leading us to hypothesize that the non-catalytic RNase III domain of KREPB5 may form an RNase III heterodimer with the endonucleases.

Bioinformatic prediction of critical amino acids in KREPB5 has therefore had limited success. The majority of targeted mutations in KREPB5 resulted in no observable defect in function in BFs (34, 35). Furthermore, the C terminus of KREPB5 has no homology to known motifs, and the divergence of *T. brucei* from other eukaryotes means that KREPB5 has no orthologs outside the kinetoplastid order. Therefore, the KREPB5 sequence cannot be used to predict and test its function. In contrast to site-directed mutagenesis, random mutagenesis does not require the *a priori* selection of residues but, rather, allows the generation and testing of many amino acid substitutions in an unbiased way. In the present study, we used random mutagenesis and an *in vivo* genetic screen to identify amino acids that are critical for KREPB5 function. We prepared and screened a library consisting of full-length KREPB5 mutant alleles for those that could not complement for the loss of KREPB5 in BFs. Numerous mutations had no effect on growth. However, seven mutations that resulted in single amino acid substitutions within the degenerate RNase III domain were found, and two were found in the C-terminal region. Alteration of these residues resulted in inhibition of growth, loss of 20S editosomes, and inhibition of *in vivo* RNA editing. Most of the same substitutions did not have the same inhibitory effects on RNA editing in PF parasites. These substitutions considerably expand the known number of single amino acid changes within editosome proteins that have differential effects on editing between life cycle stages (34). This work reveals that this phenomenon is not limited to a few specific residues but is observed for many different residues throughout multiple domains of KREPB5. The results support the existence of intrinsic functional differences between BF and PF editosomes and the involvement of KREPB5 in the control of differential editing between life cycle stages.

Overall, we analyzed hundreds of protein variants in parallel in *T. brucei* by an efficient and effective method that identified amino

acid residues that are important for protein function. This approach is particularly useful for an early-branching eukaryote, such as *T. brucei*, where sequence homology can be of limited value in predicting the position of critical residues that could be targeted by site-directed mutagenesis.

MATERIALS AND METHODS

Growth of cells *in vitro*. BF cells were grown in HMI-9 medium (59) with 10% fetal bovine serum (FBS) at 37°C in 5% CO₂. PF cells were grown in SDM-79 medium (60) with 10% FBS at 27°C. For standard growth curve analysis, cell density was measured using a Coulter counter. BF cultures were reseeded at 2×10^5 cells/ml in 10 ml every day, while PF cultures were reseeded at 2×10^6 cells/ml in 10 ml every 2 days. Unless otherwise stated, the concentrations of drugs used for selection and tetracycline (Tet)-regulated expression of transgenes in this study were as follows: for BFs, 2.5 μ g/ml G418, 5 μ g/ml hygromycin, 2.5 μ g/ml phleomycin, 0.5 μ g/ml Tet, and 0.1 μ g/ml puromycin; for PFs, 15 μ g/ml G418, 25 μ g/ml hygromycin, 2.5 μ g/ml phleomycin, 0.5 μ g/ml Tet, 1 μ g/ml puromycin, and 10 μ g/ml blasticidin.

Error-prone PCR for mutant library generation. Error-prone PCR-mediated mutagenesis of KREPB5 was performed using a GeneMorph II random mutagenesis kit (Agilent Technologies). The template plasmid was the Gateway expression clone pHD1344tub(PAC)-KREPB5-Cterm3V5, which contains the wild-type (WT) KREPB5 open reading frame without the stop codon flanked at the 5' by an *attB1* site and at the 3' by an *attB2* site (34). Briefly, 200 ng of target wild-type KREPB5 DNA (~ 1.12 μ g template plasmid) was mutagenized by 30 cycles of PCR according to the manufacturer's protocol using the following primers: *attB1* (5'-ACAAG TTTGTACAAAAAGCAG-3') and *attB2* (5'-ACCACTTTGTACAAGA AAGCT-3') (see Table S8 in the supplemental material). PCR products were separated by gel electrophoresis and purified using a gel extraction kit (Qiagen).

BP reaction for library transfer into pDONR-Express. ORFs mutagenized by PCR were transferred into pDONR-Express (37) using the BP Clonase II enzyme mix (Life Technologies). pDONR-Express (225 ng), gel-purified PCR product (150 ng), BP Clonase II enzyme mixture (3 μ l), and Tris-EDTA (TE) to 10 μ l were combined, and the mixture was incubated at room temperature (25°C) for 20 h. The reaction was stopped by adding proteinase K (1 μ l/10 μ l of the reaction volume) and incubating at 37°C for 10 min.

Full-length KREPB5 allele selection and pENTR-Express allele library isolation. Six transformations of 1.5 μ l BP reaction mixture into 80 μ l TOP10 electrocompetent cells (Life Technologies) were carried out using a BTX ECM 630 electroporator (the settings were 1,700 V, 200 M Ω , and 25 μ F). Cells from each transformation were recovered for 1 h in 1 ml SOB (61) plus 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at 37°C and 250 rpm. An aliquot of the transformation was serially diluted and plated to determine the titer of kanamycin-resistant (Kan^r) colonies, while the remaining transformants were stored as a glycerol stock. The optimal kanamycin concentration for selection of full-length KREPB5 in the pDONR-Express system was determined as previously described (37) and was 40 μ g/ml. LB plates containing 40 μ g/ml kanamycin and 1 mM IPTG were incubated at 30°C for 36 h, and colonies were then counted to determine the titer. This number was then used to estimate how much of the transformation should be plated to obtain $\sim 1,000$ colonies/plate. The glycerol stock was then thawed on ice and plated out to a density of 1,000 colonies/plate on the appropriate number of plates to produce an overall number of $\sim 25,000$ Kan^r colonies. Plates were incubated at 30°C for 36 h, all colonies were scraped from the plates, and plasmid DNA was isolated using a QIAfilter midiprep kit (Qiagen). We refer to this plasmid library of full-length mutant KREPB5 alleles as the pENTR-Express allele library.

LR reaction for transfer of the mutant allele library into a plasmid for constitutive expression in *T. brucei*. Five hundred nanograms of the destination vector pHD1344tub(PAC)/GW-Cterm3V5 (34), 250 ng of pENTR-Express containing the mutant KREPB5 allele library, 2 μ l of the

LR Clonase II enzyme mixture (Life Technologies), and TE to 10 μ l were incubated at room temperature (25°C) for 20 h. The reaction was stopped by adding proteinase K (1 μ l/10 μ l of the reaction volume) and incubating at 37°C for 10 min. The resulting expression vector encoding mutant KREPB5 (mutKREPB5) was pHD1344tub(PAC)-mutKREPB5-Cterm3V5. This plasmid contains the puromycin resistance-selectable marker (PAC) and allows constitutive expression of mutant library alleles tagged with three tandem V5 epitopes at the location corresponding to the C terminus (Cterm3V5) in the β -tubulin locus.

pHD1344tub-mutKREPB5-Cterm3V5 allele library isolation. The LR reaction mixture (1.5 μ l) was transformed into 80 μ l of TOP10 electrocompetent cells (Life Technologies) as described above. Cells were recovered for 1 h in 1 ml of SOB medium at 37°C and 250 rpm. Serial dilutions were performed and plated to determine the titer, with the remainder of the transformants being stored as a glycerol stock. The plates were incubated at 37°C for 20 to 24 h. After the titer was determined, the glycerol stock was thawed on ice and plated out to a density of 1,000 colonies/plate on the appropriate number of LB-ampicillin (100 μ g/ml) plates to produce an overall number of $\sim$ 25,000 ampicillin-resistant colonies. The plates were incubated at 30°C for 36 h, all colonies were scraped from the plates, and plasmid DNA was isolated using the QIAfilter mid-prep kit (Qiagen).

Protocol for conducting screens. Parental BF KREPB5 conditional null (CN) cells (33) and library-transfected cells were grown in 5 ng/ml Tet throughout the transfection and screening unless otherwise stated. This is the minimum concentration of Tet required for wild-type cell growth and was determined by the growth of CN cells in a range of Tet concentrations (0, 1, 2, 5, 10, 50, 500 ng/ml) (see Fig. S8 in the supplemental material). Ten micrograms of the pHD1344tub(PAC)-mutKREPB5-Cterm3V5 plasmid containing the mutant library was linearized with NotI and transfected into 3×10^7 BF KREPB5 CN cells in a volume of 100 μ l using the Amaxa Nucleofector reagent (Lonza) (38). Following transfection, the cells were diluted into 300 ml HMI-9 medium containing 2.5 μ g/ml G418, 5 μ g/ml hygromycin, 2.5 μ g/ml phleomycin, and 5 ng/ml Tet, and 1 ml was plated into each well of 24-well plates. The cells were allowed to recover for 24 h before selection in 0.1 μ g/ml puromycin. Five such transfections of the mutant library were carried out with a transfection efficiency of approximately 6×10^{-6} . Following 5 days of selection, a total of 960 individual puromycin-resistant cell lines were arrayed in 10 96-well plates. Twenty microliters of each cell line was transferred into 200 μ l of HMI-9 medium containing 2.5 μ g/ml G418, 5 μ g/ml hygromycin, 2.5 μ g/ml phleomycin, 0.1 μ g/ml puromycin, and 5 ng/ml Tet. Cells were grown to a density of approximately 2×10^5 cells/ml and then replica plated by transfer of 2 μ l into 200 μ l of HMI-9 medium that either contained 5 ng/ml Tet or completely lacked Tet. The dilution into medium lacking Tet was designed to reduce the Tet concentration to a concentration below the minimum required for cell growth. Cells were grown in the presence or absence of Tet for a total of 6 days, having been replated and diluted on day 3 to ensure that cells stayed in the logarithmic growth phase. After 6 days, cells were incubated with 20 μ l alamarBlue (Life Technologies) for 4 h to assay cell viability (39). The alamarBlue dye is a redox indicator that yields a colorimetric change (from dark blue to bright pink for healthy, proliferating cells) and a fluorescent signal in response to metabolic activity. Fluorescence measurements were obtained using a SpectraMax M2 microplate reader (Molecular Devices) with excitation at 544 nm and emission at 590 nm (590-nm cutoff). The fluorescent signal and the degree of color change are proportional to the cell density and were used to assess the viability of individual cell lines in the presence or absence of Tet. Those cells that survived in the presence of Tet but displayed a growth defect in the absence of Tet contained a mutant KREPB5 allele that could not complement the loss of KREPB5 upon Tet withdrawal. All 128 resulting cell lines with a moderate or severe growth defect in the absence of Tet were subcultured from the relevant plates containing Tet into two 96-well plates. Ninety-six cell lines without a growth defect were also subcultured in one 96-well plate. The cells from these plates were

replica plated for a second time in HMI-9 medium that lacked Tet in order to confirm the observed growth phenotypes.

PCR amplification and sequencing of library alleles from screened cell lines. Crude DNA extraction, PCR amplification, purification, and sequencing were carried out in a 96-well format. Approximately 2×10^4 cells in 10 μ l were lysed in 20 μ l lysis solution for blood (catalog number L3289; Sigma-Aldrich) at 75°C for 5 min. Neutralization solution for blood (180 μ l; catalog number N9784; Sigma-Aldrich) was then added, and samples were stored at 4°C. A total of 2.5 μ l of each sample was used for PCR amplification of the library KREPB5 sequences in the β -tubulin locus of each corresponding cell line. The forward PCR primer (primer 5355) annealed 5' of the plasmid integration site in the genomic β -tubulin locus, and the reverse primer (primer 6931) annealed in the coding sequence for the C-terminal V5 tag in the pHD1344tub(PAC)-mutKREPB5-Cterm3V5 plasmid (see Table S8 in the supplemental material). The expected product size for a correctly integrated construct was $\sim$ 2.3 kb. The PCR products were visualized using agarose gel electrophoresis and sequenced directly with two nested sequencing primers to cover the entire KREPB5 coding sequence. The forward sequencing primer (primer 9571) annealed in the GPEET splice acceptor site, and the reverse primer (primer 10622) annealed in the coding sequence for the C-terminal V5 tag, and both of these sites flanked the library KREPB5 sequence in the pHD1344tub(PAC)-mutKREPB5-Cterm3V5 plasmid (see Table S8 in the supplemental material).

WT G121R control for screening. As a control for the screening procedure, we mixed equal amounts of the pHD1344tub(PAC)-KREPB5-Cterm3V5 plasmid containing the WT KREPB5 ORF with pHD1344tub(PAC)-G121R-KREPB5-Cterm3V5, which contains the KREPB5 ORF encoding the previously characterized G121R mutation (34, 35). Ten micrograms of the plasmid mixture was linearized with NotI and transfected into 3×10^7 BF KREPB5 CN cells, as described above for the mutant library transfection. Ninety-six individual puromycin-resistant cell lines were arrayed in a 96-well format and screened as described above.

Generation of exclusive expression cell lines. The Gateway expression clone pHD1344tub(PAC)-KREPB5-Cterm3V5 (34) was used as a template for site-directed mutagenesis (QuikChange II kit; Agilent) using the forward and reverse primers listed in Table S8 in the supplemental material. NotI-digested plasmids were transfected into the relevant BF (33) and PF (34) KREPB5 CN cells. Transfections of BF cell lines with the Amaxa Nucleofector (Lonza) and of PF cell lines with the BTX transfection device (Harvard Apparatus, Inc.) were carried out as described by Merritt and Stuart (38). Cell lines resistant to puromycin were selected, and constitutive expression of KREPB5-3 $\times$ V5 was confirmed by Western blotting.

Fractionation on glycerol gradients. Glycerol gradient fractionation of PF cells was carried out on crude mitochondrial preparations (40) from 2×10^9 PF or BF cells in the presence or absence of 0.5 μ g/ml Tet. Following lysis in 650 μ l lysis buffer (10 mM Tris-HCl [pH 7.2], 10 mM MgCl₂, 100 mM KCl, 1% Triton X-100) and centrifugation (13,000 rpm, 10 min, 4°C), cleared lysates were loaded onto 11-ml 10 to 30% glycerol gradients and centrifuged at 38,000 rpm for 5 h or 9 h at 4°C in an SW 40 Ti rotor (Beckman). Twenty-four fractions of 500 μ l each were collected from top to bottom, flash frozen in liquid nitrogen, and stored at -80°C .

Immunoprecipitation. Cleared lysate was prepared by lysis of 2×10^8 cells in 1 ml IPP150 (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40) with 1% Triton X-100, followed by centrifugation at 10,000 $\times$ g and 4°C. For each immunoprecipitation, 0.5 ml cleared lysate (1×10^8 cells) was incubated overnight with 1 μ l of rabbit antibody (Rockland Immunochemicals) (see Table S9 in the supplemental material) specific for the V5 epitope tag. Magnetic beads (12.5 μ l; Protein G Mag Sepharose Xtra; GE Healthcare) were washed twice with 1 ml of 1 $\times$ phosphate-buffered saline–0.1% bovine serum albumin and once with 1 ml IPP150. The beads were then incubated for 4 h with rotation at 4°C with cleared lysate/antibody. After incubation, the supernatant was removed and the beads

were washed four times with 1 ml of IPP150. Complexes bound to beads were eluted by heating with 100 μ l of 2 $\times$ SDS sample buffer for 5 min at 95°C.

SDS-PAGE and Western blotting. SDS-PAGE loading buffer was added to samples containing purified protein complexes and resolved on 10% SDS-polyacrylamide gels (Criterion Tris-HCl; Bio-Rad). For Western analysis, resolved proteins were transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore) and probed using monoclonal antibodies against KREPA1, KREPA2, KREL1, and KREPA3 as previously described (21) (see Table S9 in the supplemental material). Blots were sequentially stripped and reprobed using mouse monoclonal primary antibody against the V5 epitope tag at 1:5,000 with goat anti-mouse immunoglobulin secondary antibody conjugated with horseradish peroxidase at 1:5,000 (see Table S9 in the supplemental material). Blots were developed with an enhanced chemiluminescence kit (Thermo Scientific) per the manufacturer's instructions and imaged using a FluorChem E system (ProteinSimple) or X-ray film (Kodak). Positive-control 20S samples from purified PF mitochondria (IsTaR 1.7a strain) were generated as previously described (27, 41).

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR) analysis. Total RNA was harvested from BF and PF cell lines using the TRIzol reagent and treated with Turbo DNase (both from Life Technologies) according to the manufacturer's instructions. RNA integrity was confirmed using an RNA nanochip on a Bioanalyzer (Agilent Technologies). Two micrograms of total RNA was reverse transcribed using TaqMan reverse transcription reagents and MultiScribe reverse transcriptase (Life Technologies). The abundances of reference, never-edited, pre-edited, and edited transcript cDNAs were then analyzed by high-throughput real-time PCR as described by McDermott et al. (34). cDNAs were first preamplified in multiplex specific-target-amplification (STA) reactions using TaqMan PreAmp master mix (Life Technologies). STA reactions were performed with the following thermocycling conditions: 1 cycle at 95°C for 10 min and 14 cycles of 95°C for 15 s and 60°C for 4 min. Preamplified cDNA was treated with exonuclease I (New England Biolabs) and diluted 10-fold. High-throughput real-time PCR was then conducted on the BioMark HD system, using SsoFast EvaGreen supermix with Low ROX (Bio-Rad) and Fluidigm 48-by-48 dynamic array integrated fluidic circuits (IFCs). The primers used are described in Table S8 in the supplemental material. The same primers were used for both the STA reaction and real-time PCRs. All samples were assayed in quadruplicate. Processing of the IFCs and operation of the instruments were performed according to the manufacturer's procedures. PCR was performed using the thermal protocol GE Fast 96 $\times$ 96 PCR + Melt (v2.pcl). Data were analyzed using Fluidigm real-time PCR analysis software, the linear (derivative) baseline correction method, and the auto (global) threshold cycle (C_T) method. The C_T values determined were exported to Excel software for further processing. The fold changes in mRNA levels in samples following Tet withdrawal relative to the mRNA levels in the presence of Tet were calculated using the average C_T value for the quadruplicate samples and the $2^{-\Delta\Delta C_T}$ method (42). The average standard deviation of replicate values was ≤ 0.09 for all samples.

Homology modeling. The comparative model of the RNase III domain of KREPB5 was described previously (34) and was generated using the HHPred (43) and Modeler (44) programs. Swiss-Model (45) was used as a tool to independently verify the model created by Modeler.

RESULTS

Generation of a KREPB5 mutant allele library. To identify amino acids critical for KREPB5 function, we generated a library of mutant KREPB5 alleles using error-prone PCR. To reduce base substitution bias and to increase diversity, we used the commercially available GeneMorph II kit, which uses a combination of two DNA polymerases with opposing mutational spectra. The mutation frequency per mutant allele can be described as the product of the DNA polymerase error rate and the number of

amplifications, such that the more often that a target is replicated, the more errors that are accumulated. Therefore, the initial DNA amount was used to adjust the mutation frequency. Low mutation rates result in libraries with many functional sequences but small numbers of total mutations and low coverage. High mutation rates enrich libraries for sequences with high numbers of mutations, leading to good coverage and fewer functional sequences. However, the accumulation of mutations leads to a decrease in the number of sequences containing unique single mutations. As the majority of targeted KREPB5 mutants with single amino acid changes had no observable defect in function, we aimed for an intermediate mutation rate to balance coverage, functional retention, and uniqueness.

ORFs mutagenized by PCR were placed in frame with a kanamycin resistance-encoding gene in the entry vector pDONR-Express (37). Transformation of the resulting plasmid library into *Escherichia coli* and selection on kanamycin-containing plates were used to eliminate nonsense and frameshift mutations (Fig. 1A). This enriched the library with full-length ORFs, which were then transferred into the Gateway destination vector pHD1344tub(PAC)-3V5GW, which allows constitutive expression of the encoded protein with a C-terminal 3 $\times$ V5 epitope tag from the β -tubulin locus in *T. brucei*. Approximately 25,000 individual plasmids were present in the library. Forty-eight KREPB5 alleles were sequenced to assess mutational bias, diversity, coverage, and the unique mutation rate. A total of 124 mutations were identified, and of these, 119 were distinct. There was an average of ~ 2.5 mutations per ORF, while the modal number of mutations observed per ORF was 3, present in 15/48 sequences. Four of 48 sequenced alleles were wild-type (WT) nucleotide sequences with no mutations (see Fig. S1A in the supplemental material). All mutated ORFs had distinct nucleotide sequences. The sequenced ORFs were translated *in silico* to assess the effects of the mutations on the protein sequence. No nonsense or frameshift mutations were identified, indicating that selection on kanamycin had successfully enriched for ORFs encoding full-length proteins. A total of 46/124 mutations were silent, and a total of 12/48 (25%) ORFs had no mutations corresponding to amino acid changes and thus encoded the WT protein. The remaining 36 ORFs contained a total of 76 distinct missense mutations. These 76 missense mutations resulted in a total of 75 different changes in 71 amino acid positions in the protein sequence. The proteins encoded by these 36 ORFs had an average of ~ 2.1 amino acid changes per protein. Most contained 2 changes per protein (14/36; $\sim 39\%$), with a high proportion also containing just 1 amino acid change (10/36; $\sim 28\%$) (see Fig. S1B and C in the supplemental material). Thus, the KREPB5 sequences isolated from this library indicated that the library was diverse and had a satisfactory balance of unique mutation rate and coverage. The proportion of sequences that had either no mutations or only silent mutations indicated that there would be no additional advantage to using a lower rate of mutagenesis.

Isolation of KREPB5 alleles in the mutant library that do not complement the loss of KREPB5. To screen for mutant KREPB5 alleles that could not complement the loss of KREPB5, we transfected the library of full-length mutant alleles into BF KREPB5 conditional null (CN) cells (33). Both endogenous alleles have been deleted in these cells, and a tetracycline (Tet)-regulatable WT KREPB5 allele has been inserted into the rRNA locus. BF cells were used for the ease of obtaining clonal cell lines following transfection.

FIG 1 Identification of mutated KREPB5 alleles that do not complement for the loss of KREPB5 in BFs. (A) Diagram showing that KREPB5 ORFs were mutagenized (mutations are represented by asterisks), placed in frame with a kanamycin resistance (Kan^r) gene in the pDONR-Express entry vector (37), selected in *E. coli* on kanamycin plates to eliminate nonsense and frameshift mutations, and transferred into the pHD1344tub(PAC)-3V5GW *T. brucei* expression vector by the Gateway reaction. White boxes, Gateway recombination sites; epPCR, error-prone PCR. (B) Schematic of the genetic complementation assay used to identify the loss of KREPB5 function mutations in BFs. The mutant plasmid library shown in panel A was transfected into BF KREPB5 CN cells, and puromycin-resistant transfected clones were selected, arrayed in 96-well plates, replica plated into medium that contained or lacked Tet, and incubated with alamarBlue after 6 days of growth. Pink and blue, cell proliferation and growth inhibition, respectively. Cells that grow in the presence of Tet but not in its absence contain a mutant KREPB5 allele that does not complement the loss of KREPB5. See Materials and Methods for details.

tion. After transfection and selection, 960 cell lines were arrayed in 96-well plates and grown in the presence or absence of Tet for 6 days. In the absence of Tet, expression of the regulatable WT KREPB5 was repressed, and thus, the cells exclusively expressed the mutant library alleles. Cells containing functional alleles survived, while cells containing nonfunctional alleles had a growth defect. To assess cell viability in the presence or absence of Tet, we incubated cells with alamarBlue (39). The alamarBlue dye yields a colorimetric change and a fluorescent signal in response to meta-

bolic activity. Cell lines that survived in the presence of Tet (pink, highly fluorescent wells) but had a growth defect in the absence of Tet (purple or blue wells) contained a mutant KREPB5 allele that could not complement the loss of KREPB5 (Fig. 1B; see also Fig. S2A and Tables S1 and S2 in the supplemental material). In this way, a total of 128 cell lines (~13%) were observed to have a growth defect in the absence of Tet (see Table S3 in the supplemental material). As a control for the screening procedure, we mixed equal amounts of the pHD1344tub(PAC)-KREPB5-

TABLE 1 Numbers of amino acid changes in distinct KREPB5 variants that did not complement the loss of KREPB5^a

No. of amino acid changes encoded per ORF	No. of distinct ORFs observed	No. of ORFs where a mutation resulting in a single amino acid change can explain growth defect
1	8	8
2	16	7
3	31	4
4	17	6
5	14	3
6	11	3
7	7	1
8	2	1
9	1	0
Total	107	33

^a A single mutation at 1 of 8 sites may explain the lack of complementation in 31% of the 107 noncomplementing ORFs.

Cterm3V5 plasmid containing the WT KREPB5 ORF with pHD1344tub(PAC)-G121R-KREPB5-Cterm3V5, which contains the KREPB5 ORF encoding the previously characterized lethal G121R substitution. This plasmid mixture was transfected into BF KREPB5 CN cells, and 96 cell lines were screened in the same way as cells containing mutant library alleles. Sixty-five cell lines survived in the absence of Tet, while 31 had a growth defect. Genomic DNA was isolated from these cell lines, and the sequence of the KREPB5 alleles present in the β -tubulin locus was obtained. As expected, sequencing revealed that cells with a growth defect contained a KREPB5 allele with the G121R mutation, and those that survived contained a WT KREPB5 allele. These results indicate that a genetic selection in *T. brucei* CN BF cells containing the WT KREPB5 ORF can efficiently isolate nonfunctional KREPB5 alleles.

Identification of amino acid substitutions in nonfunctional and functional KREPB5 variants. We obtained the sequences of nonfunctional KREPB5 alleles by isolating genomic DNA from the 128 cell lines containing mutant library alleles that displayed a growth defect. The KREPB5 alleles in the β -tubulin locus for each of these cell lines were amplified by PCR and sequenced. A PCR product was not obtained for five clones, indicating that these cell lines did not have the intended integration of a mutant library allele into the β -tubulin locus (see Fig. S2B in the supplemental material). Sequence data with full coverage of the KREPB5 allele were acquired for 120 cell lines. Three sequences contained nonsense mutations that were not eliminated from the library by kanamycin selection and were discounted from further analysis. A further 10 sequences were duplicates of others, resulting in a total of 107 distinct nonfunctional KREPB5 sequences containing missense mutations. Eight ORFs each encoded only one unique amino acid change (Tables 1 and 2; see also Table S4 in the supplemental material): R77L, V93E, S97T, F114S, G121R, L134P, V152E, or S320P. Seven of these deleterious substitutions (R77 to V152) were in residues within the degenerate RNase III domain and include the previously described substitutions R77L, F114S, and G121R (34, 35) (Table 2). The remaining S320P substitution lies in the C-terminal region that has no detectable homology to known motifs. The majority of alleles from cell lines with growth defects encoded proteins with multiple amino acid changes, rang-

TABLE 2 Mutations of KREPB5 that result in a growth defect

Single change	No. of ORFs encoding change of essential residue	Substitution(s) identified in ORFs encoding multiple residue changes
R77L	1	R $\rightarrow$ Q
V93E	4	V $\rightarrow$ E, V $\rightarrow$ M, V $\rightarrow$ A
S97T	1	S $\rightarrow$ C
F114S	1	F $\rightarrow$ S
G121R	4 (G121E and V152M in 1 clone)	G $\rightarrow$ V, G $\rightarrow$ E
L134P	2	L $\rightarrow$ P
V152E	7 (G121E and V152M in 1 clone)	V $\rightarrow$ E, V $\rightarrow$ M, V $\rightarrow$ A
S320P	6	S $\rightarrow$ F, S $\rightarrow$ Y, S $\rightarrow$ P
Total	25 ^a	

^a One clone had both G121R and V152M substitutions. See the text for details.

ing from two up to nine changes. Most nonfunctional alleles encoded proteins with three amino acid changes (Table 1; see also Table S4 in the supplemental material), and the average number of substitutions per nonfunctional protein was $\sim$ 3.8. A proportion of ORFs with multiple substitutions encoded changes to the same essential residues described above. Indeed, the residues V152, S320, V93, and G121 were the most frequently substituted residues encoded by all nonfunctional ORFs (Table 2; see also Table S4 in the supplemental material). This can therefore explain the growth defect that occurs upon exclusive expression of these ORFs, if we assume that amino acid substitutions that are observed at a particular residue have the same functional effects. For example, if V152M and V152A, in addition to V152E, are nonfunctional, this explains the growth defect observed in seven cell lines (Tables 1 and 2; see also Table S4 in the supplemental material). The growth defects of 33/107 ($\sim$ 31%) individual cell lines were accounted for in this way (Table 1; see also Table S4 in the supplemental material).

PCR products were also obtained for 96 randomly chosen cell lines that did not have a growth defect, and these yielded 93 distinct sequences. Twenty of these sequences ($\sim$ 21%) contained no mutations or only silent mutations and therefore encoded the WT protein (Table 3; see also Table S5 in the supplemental material). This is consistent with the proportion of sequences encoding the WT protein in the initial analysis of a small subset of library clones (see Fig. S1B in the supplemental material). The remaining alleles that did not affect KREPB5 function encoded a total of 170 amino acid changes, including 159 distinct substitutions. Three of these

TABLE 3 Numbers of amino acid changes in distinct KREPB5 variants that complemented the loss of KREPB5^a

No. of changes	No. of distinct ORFs
0	20
1	22
2	26
3	13
4	5
5	5
6	1
7	1
Total	93

^a Twenty of 93 ($\sim$ 21%) ORFs were either fully wild type or contained only silent mutations.

TABLE 4 Single substitutions in ORFs with multiple mutations predicted to not complement the loss of KREPB5 on the basis of subtraction of mutations that allowed complementation

Residue	Observed amino acid change	KREPB5 domain
C63	C → G	
L161	L → Q	RNase III
K168	K → E	RNase III
A169	A → P	RNase III
V197	V → L	RNase III
V197	V → D	RNase III
A209	A → G	
L235	L → F	PUF
R253	R → G	C terminal
E256	E → D	C terminal
W258	W → G	C terminal
W258	W → C	C terminal
T321	T → I	C terminal
L323	L → Q	C terminal
R370	R → K	C terminal

substitutions, E122D, S123N, and C232S, were in residues that had previously been targeted by mutation without any defects in cell growth (34, 35). Most functional alleles (~28%) encoded proteins with two amino acid changes (Table 3; see also Table S5 in the supplemental material), and a high proportion (~24%) also encoded proteins with single amino acid substitutions. The average number of substitutions per functional protein was ~1.8, which is lower than the average number of substitutions per nonfunctional protein. However, some alleles encoded functional KREPB5 proteins that had up to seven substitutions (Table 3; see also Table S5 in the supplemental material). These replacements can be non-conservative and found across the whole protein, indicating that KREPB5 can tolerate a high number of substitutions and yet still retain function (see Table S5 in the supplemental material).

In all products of sequenced nonfunctional and functional ORFs, 293 of the 384 (~76%) residues in KREPB5 were changed to a different amino acid. In products of nonfunctional ORFs, a total of 245 individual residues were changed to a different amino acid, while in products of functional ORFs that were sequenced,

134 individual residues were changed. Eighty-six residues were replaced in products of both nonfunctional and functional ORFs (see Table S6 in the supplemental material). No substitutions at R77, V93, S97, F114, G121, and V152 and only highly conservative changes at L134 and S320 were observed in products of functional ORFs. By subtraction of the number of residues affected in products of functional ORFs from the number of residues changed in those of nonfunctional ORFs, we were able to deduce a further 13 single amino acid substitutions in the KREPB5 RNase III domain and C-terminal region predicted to result in a nonfunctional protein (Table 4). These predictions assume that the loss of function is derived from a single amino acid change and do not account for amino acids functioning in concert. These predictions also ignore the nature of the amino acid change; e.g., L349H and L349R are treated equally. Experimental validation of these predictions is therefore needed to determine if more complex amino acid interactions cause the loss of function in alleles with multiple substitutions.

Comparison of functional data to evolutionary conservation. Conservation analysis is a widely used method for predicting important residues in protein sequences, as the mutational sensitivity and the evolutionary conservation of a residue are strongly correlated (46). To study the correlation between KREPB5 conservation and function as defined by our screen, we compared the degree of conservation for each KREPB5 position across all kinetoplastids. We used Jensen-Shannon divergence scoring (47) for each amino acid position in a MUSCLE (48) multiple-sequence alignment of 29 KREPB5 sequences from a wide range of kinetoplastid species (see Fig. S3 and Table S7 in the supplemental material). Most of the amino acids that were affected in non-functional *T. brucei* KREPB5 proteins with single deleterious substitutions were highly conserved across all kinetoplastids (Table 5). The exceptions to this were S97 and V152. *T. brucei* S97 aligns with glycine residues in *Trypanosoma cruzi*, the majority of *Leishmania* sequences, and *Bodo saltans*. V152 aligns with isoleucine in *T. cruzi* CL Brener; methionine in *Trypanosoma grayi*, all other *T. cruzi* strains, and *Leishmania* species; leucine in *Crithidia fasciculata*; and cysteine in *B. saltans* (Table 5). Thus, S97 and V152 would have been overlooked if functionally important residues

TABLE 5 Alignment of noncomplementing KREPB5 amino acid substitutions with the corresponding residues in 29 kinetoplastid species

Alignment column no.	Divergence score ^a	Sequence in the alignment for species 1 to 29 ^b	<i>T. brucei</i> Lister 427 residue	Substitution
115	0.754	RRRRRRRRR-RRRRRRRRRRRRRRRRRR	R77	R → L
131	0.799	VVVVVVVVV-VVVVVVVVVVVVVVVVVVV	V93	V → E
135	0.717	SSSSSSSGG-GGGSGGGGGGGSGSSSSG	S97	S → T
152	0.770	FFFFFFFFF-FFFFFFFFFFFFFFFFFFFF	F114	F → S
159	0.737	GGGGGGGGG-GGGGGGGGGGGGGGGGGG	G121	G → R
172	0.728	LLLLLLLLL-LLLLLLLLLLLLLLLLLLLL	L134	L → P
190	0.624	VVVVVVIMMMMMMMMMMMMMMMMMMLC	V152	V → E
369	0.717	SSSSSSSSSSSSSSSSSSSSSSSSSSST	S320	S → P
Mean score	0.731			
SD score	0.052			

^a Jensen-Shannon divergence score for the position corresponding to the indicated *T. brucei* residue.

^b The order of the species is as follows: 1, *T. brucei* Lister 427; 2, *T. brucei* TREU927; 3, *T. brucei gambiense*; 4, *Trypanosoma evansi*; 5, *Trypanosoma vivax*; 6, *T. grayi*; 7, *Trypanosoma congolense*; 8, *T. cruzi* strain CL Brener; 9, *T. cruzi* Sylvio X10; 10, *T. cruzi* Dm28c; 11, *T. cruzi marinkellei*; 12, *Leishmania* sp. strain MAR LEM2494; 13, *Leishmania enriettii*; 14, *Leishmania tarentolae*; 15, *Leishmania braziliensis* MHOM/BR/75/M2903; 16, *L. braziliensis* MHOM/BR/75/M2904; 17, *Leishmania panamensis*; 18, *Leishmania mexicana*; 19, *Leishmania donovani*; 20, *Leishmania infantum*; 21, *Leishmania aethiopica*; 22, *Leishmania tropica*; 23, *Leishmania arabica*; 24, *Leishmania major*; 25, *Leishmania gerbilli*; 26, *L. gerbilli*; 27, *Leishmania turanica*; 28, *Crithidia fasciculata*; 29, *Bodo saltans*.

TABLE 6 Alignment of complementing KREPB5 amino acid substitutions with the corresponding residues in 29 kinetoplastid species

Alignment column no.	Divergence score ^a	Sequence in the alignment for species 1 to 29 ^b	<i>T. brucei</i> Lister 427 residue	Substitution(s)
109	0.712	CCCCCCFF-FFFFFFFFFFFFFFFFFYS	C71	C → Y
117	0.611	GGGGSGDD-DTTTTTTTTTTTTTTSA	G79	G → D
120	0.684	HHHRRRRR-RRRRRRRRRRRRRRRS	H82	H → Y
124	0.618	SSSRGGGG-GQQQQQQQQQQQQQAH	S86	S → G
134	0.807	HHHHHHHH-HHHHHHHHHHHHHHHH	H96	H → Y
143	0.658	TTTTTTTT-TTTTTTTTTTTTTTTSA	T105	T → S
148	0.715	TTTTTTTT-TTTTTTTTTTTTTTTIV	T110	T → M
180	0.617	SSSSASASS-SYYYYYYYYYYYYYYD	S142	S → L
252	0.852	HHHHHQHHHHHHHHHHHHHHHHH	H214	H → L
253	0.844	NNNNNNNNNNNNNNNNNNNNNNN	N215	N → Y
278	0.719	AAAAAAAAAAAAAAAAAAAAAAAAAV	A240	A → V
324	0.492	KKKKCRHKKKKVVVVVVVVVVVLN	K286	K → N
339	0.486	EEEEEEEEEDDEEDDDDDDDDDQ-	E290	E → D
344	0.509	RRRRRRRRRLRILLRLLLLLLLLLI-	R295	R → W
348	0.577	AAAAAAAAAAHHHHHHHHHHHHHQS	A299	A → T
371	0.747	MMMMMMMMMMMMMMMMMMMMMMMI	M322	M → K, M → L
375	0.656	WWWFWWWWLFRFFCCCCCCCCCS	W326	W → C
378	0.697	KKKKKKKKKRRRRRRRRRRRRRK	K329	K → E
391	0.693	DDDDDDDDDDDDDDDDDDDDDK	D342	D → Y
392	0.603	KKKKTRKKKKNNNNNNNNNNNKTH	K343	K → N
406	0.562	SSSSKLSSEEEEEEEEEEEEEETS	S352	S → P
Mean score	0.660			
SD score	0.105			

^a Jensen-Shannon divergence score for the position corresponding to the indicated *T. brucei* residue.
^b See footnote *b* of Table 5 for the order of the species.

were solely predicted using strong evolutionary conservation. In general, functional KREPB5 variants with single amino acid changes contain substitutions in less-conserved residues than nonfunctional variants. However, some functional variants do tolerate substitutions in highly conserved residues (Table 6). These include H214, N215, M322, and D342, which can tolerate replacement by leucine, tyrosine, lysine, and tyrosine, respectively. These results demonstrate that sequence conservation cannot be solely used to predict function and highlights the power of the random mutagenesis approach.

Analysis of deleterious substitutions in nonfunctional KREPB5 variants. In order to confirm the growth defect of cells containing the single amino acid substitutions isolated in the complementation screen, we prepared new site-directed V93E, S97T, F114S, L134P, V152E, and S320P mutant KREPB5 allele constructs that were reinserted into the β -tubulin locus of BF KREPB5 CN cells. The cells containing WT, R77L, and G121R alleles were previously described (34). These alleles were also tagged with a C-terminal V5 epitope tag. All cells grew at the same rate as the parental BF KREPB5 CN cells in the presence of Tet, regardless of which allele was in the tubulin locus, confirming that none of the mutations had a dominant negative effect (Fig. 2A to E). Expression of the WT or mutant alleles was confirmed by Western analysis that probed for the V5 tag (Fig. 2F). Exclusive expression of WT KREPB5 in the absence of Tet resulted in normal growth, confirming that the C-terminal V5 tag did not affect function, while exclusive expression of R77L, V93E, S97T, G121R, V152E, and S320P mutant alleles resulted in severe growth inhibition (Fig. 2A to E). This is consistent with previously reported analyses of the R77L and G121R substitutions in BFs (34, 35). Exclusive expression of L134P and F114S mutant alleles resulted

in more moderate growth defects (Fig. 2C and E), consistent with the findings of a previous analysis of the F114S substitution (35). We also analyzed the effect of exclusive expression of a KREPB5 S86G mutant allele. This single substitution was encoded by a functional allele in the complementation screen but is relatively close in sequence space to the R77, V93, and S97 residues that were substituted in nonfunctional alleles. As expected, exclusive expression of the S86G mutant allele in the absence of Tet resulted in normal growth (Fig. 2A). We also tested single substitutions that were predicted to be deleterious to KREPB5 function (Table 4). W258 is highly conserved in all kinetoplastids (Table 7; see also Fig. S3 in the supplemental material), and exclusive expression of a W258G mutant allele resulted in severe growth inhibition (Fig. 2D). However, exclusive expression of the C63G, K168E, and R370K mutant alleles resulted in normal growth (data not shown). These results show that identification of deleterious amino acid substitutions can be made by subtractive deduction, albeit with the expected false-positive predictions that result from the assumptions described above.

In total, seven deleterious substitutions (R77L, V93E, S97T, F114S, G121R, L134P, and V152E) were found across the degenerate RNase III domain, while two (W258G and S320P) were found in the C-terminal region that has no detectable homology to known motifs (Fig. 3A). None were found in the degenerate U1-like zinc finger or in the PUF motif. This could be due to unique mutation rates at these residues or incomplete library coverage, but it is also consistent with the findings of previous studies that showed that these motifs are not required for KREPB5 function in BFs (34, 35). Homology modeling of the KREPB5 RNase III domain with the template *Aquifex aeolicus* RNase III (PDB accession number 2EZ6) (34) places R77, V93, and S97 away from the RNase III active site or the region responsible for

FIG 2 Confirmation that the identified KREPB5 mutations result in the growth defect in BFs. (A to E) Cumulative growth of BF CN cells that contain a constitutively expressed V5-tagged WT or mutant KREPB5 allele in the β -tubulin locus and a Tet-regulatable WT KREPB5 allele that is expressed (identified by E) in the presence of Tet. The mutant or WT alleles are exclusively expressed when the regulatable allele is repressed (identified by R) in the absence of Tet. Exclusive expression of the WT or S86G mutant proteins results in normal growth, while that of the R77L, V93E, S97T, L134P, V152E, W258G, S320P, F114S, and G121R mutant proteins results in growth defects. (F) Western analysis of BF cell lysates (2×10^6 cell equivalents per lane) with anti-V5 tag antibody showing expression of the V5-tagged WT or mutant KREPB5 proteins in cells in which the Tet-regulatable WT allele was expressed (E) or repressed (R) for 3 days.

dimerization (Fig. 3B), suggesting interaction with or proximity to other proteins. As described previously, G121 lies in the α -helical region that is important for the dimerization of RNase III domains and that creates the functional catalytic center in other RNase III endonucleases. Mutation of this glycine to arginine in KREPB5 may result in steric interference that prevents normal dimerization. F114S and L134P also lie in this region, while V152 is structurally close to residues that correspond to the catalytic center and that coordinate

the divalent metal ion cofactor in other RNase III endonucleases (Fig. 3B).

Differential effects of KREPB5 substitutions in BFs and PFs. Because we had previously identified an amino acid change (R77L) in KREPB5 that affected cell growth and RNA editing in BF cells but not PF cells (34), we tested nonfunctional mutant alleles isolated in the BF complementation screen for their ability to complement the loss of KREPB5 in PF KREPB5 CN cells (Fig. 4A to E). Expression of the WT

TABLE 7 Alignment of predicted noncomplementing KREPB5 amino acid substitutions with the corresponding residues in 29 kinetoplastid species

Alignment column no.	Divergence score	Sequence in the alignment for species 1 to 29 ^b	<i>T. brucei</i> Lister 427 residue	Predicted nonfunctional substitution(s)
101	0.724	CCCCCCCC-CSSSSSSSSSSSSSSSE	C63	C → G
199	0.644	LLLLLLLLIIIIIAAAAAAAAAAAAAAAL	L161	L → Q
206	0.640	KKKKKKKKKKKKQQQQQQQQQQQQQTN	K168	K → E
235	0.695	VVVVVVVVVVIVVVVVVVVVVVIL	V197	V → L, V → D
247	0.767	AAAAAAAAAAPPPPPPPPPPPPPPA	A209	A → G
273	0.700	LLLLLLLLLLLLLLLLLLLLLLLLLV	L235	L → F
291	0.655	RRRRRRRRRRDDDDDDDDDDDDDEE	R253	R → G
294	0.672	EEEEEEEEERRRRRRRRRRRRRRGS	E256	E → D
296	0.846	WWWWWWWWWWWWWWWWWWWWWW	W258	W → G, W → C
370	0.671	TTTTTTTKKKKFFFFFFFFFFFFFS	T321	T → I
372	0.651	LLLLRRKKKKRRRRRRRRRRRRRP	L323	L → Q
428	0.716	RRRRRRRRRRRRRRRRRRRRRRRR	R370	R → K
Mean score	0.698			
SD score	0.060			

^a Jensen-Shannon divergence score for the position corresponding to the indicated *T. brucei* residue.
^b See footnote *b* of Table 5 for the order of the species.

or mutant alleles was confirmed by a Western analysis that probed for the V5 tag (Fig. 4F). The anti-V5 antibody also bound the protein A portion of the Tet-regulatable WT KREPB5-tandem affinity purification (TAP)-tagged protein present in the PF KREPB5 CN cells, which showed that WT KREPB5-TAP is not expressed upon Tet withdrawal (Fig. 4F). Exclusive expression of WT KREPB5 and R77L, V93E, S97T, F114S, L134P, V152E, W258G, and S320P mutant KREPB5 in the absence of Tet resulted in normal PF growth (Fig. 4A to E). Only the G121R mutant did not rescue the growth defect of the parental PF KREPB5 CN cells (Fig. 4E) (34).
The effects of exclusive expression of the mutant allele on BF

and PF RNA editing *in vivo* were assessed by high-throughput RT-qPCR using the Fluidigm BioMark system. The abundances of preedited, edited, and never-edited mitochondrial mRNAs and KREPB5 mRNAs in BF and PF KREPB5 CN cells that were exclusively expressing WT or mutant alleles relative to those in the corresponding cells in which the WT Tet-regulatable KREPB5 allele was also expressed were determined. Parallel to the effects on cell growth, exclusive expression of WT or S86G mutant KREPB5 rescued the *in vivo* editing defects observed in the parental BF and PF KREPB5 CN cells (Fig. 5; see also Fig. S4 to S6 in the supplemental material). Exclusive ex-

FIG 3 Locations of residues that are essential for KREPB5 function in BFs. (A) Schematic showing the locations (asterisks) of the loss-of-BF-function substitutions in predicted and characterized motifs (34, 35). U1-ZnF, degenerate U1-like C₂H₂-type zinc finger; RNase III, degenerate RNase III domain; PUF, Pumilio and fem-3 binding factor motif. (B) Ribbon representations of the RNase III domain of KREPB5 based on comparative modeling with the template *Aquifex aeolicus* RNase III complexed with its product of double-stranded RNA processing (PDB accession number 2EZ6). Dark gray, KREPB5 structure; light gray, template RNase III domain and dsRNA binding domain; green, RNA; magenta, the positions of deleterious single amino acid substitutions in the KREPB5 RNase III domain.

FIG 4 Only one of the mutant KREPB5 alleles that inhibits growth in BFs does so in PFs. (A to E) Cumulative growth of PF KREPB5 CN cells that express WT or mutant versions of KREPB5 in cells in which the Tet-regulatable WT KREPB5 allele was expressed (indicated by E) or repressed (indicated by R). See the legend for Fig. 2. Exclusive expression of the G121R mutant protein resulted in a growth defect in PFs, while that of WT or R77L, V93E, S97T, L134P, V152E, W258G, S320P, or F114S mutant proteins did not. (F) Western analysis of PF cell lysates (2×10^6 cell equivalents per lane) that contained a V5-tagged WT or mutant KREPB5 allele in the β -tubulin locus and a TAP-tagged Tet-regulatable WT allele that was expressed (E) or repressed (R) for 4 days. The blots were probed with an anti-V5 antibody that also binds the protein A portion of the TAP tag.

pression of mutant alleles encoding proteins with R77L, V93E, S97T, F114S, G121R, L134P, V152E, W258G, and S320P substitutions resulted in reduced abundances of edited transcripts in BFs (Fig. 5A). Different substitutions had different effects on editing *in vivo*. For example, in BF cells exclusively expressing L134P mutant KREPB5, the amount of reduction in edited mRNAs ranged from ~63% (COIII, MURF2, and RPS12) to >99% (ND3) (Fig. 5B). Cells that exclusively expressed the

V152E mutant KREPB5 had a >99% reduction in the amounts of all edited mRNAs assayed (Fig. 5B). The smaller effect of the L134P substitution on RNA editing *in vivo* is consistent with the more moderate growth defect of cells exclusively expressing this protein variant (Fig. 2C). In all cases except the case involving the G121R substitution, the substitutions did not have the same inhibitory effects on RNA editing in PF parasites (Fig. 5; see also Fig. S4 to S6 in the supplemental material). For

example, in PF cells exclusively expressing L134P or V152E mutant KREPB5, the greatest observed reduction in any edited mRNA assayed was ~50% (Fig. 5B), which was not enough to impact cell growth (Fig. 4A to E). As previously reported (34), there was a strong inhibition of *in vivo* editing by the G121R substitution in PFs, which correlates with the severe growth defect of cells exclusively expressing this protein variant (Fig. 4E).

The effects of these mutations on editosomes in BF and PFs were also evaluated by immunoprecipitation of V5-tagged mutant KREPB5 and by glycerol gradient sedimentation of mitochondrial lysates. Immunoprecipitation with anti-V5 antibodies showed almost no coimmunoprecipitation of editosome components by all mutants expressed by BF cells (Fig. 6A). BF cells that exclusively expressed these mutant alleles had a much-reduced level of editosome components compared to that for cells expressing Tet-regulatable WT KREPB5 or cells exclusively expressing WT KREPB5 from the β -tubulin locus (see Fig. S7A in the supplemental material). In contrast, for PFs editosome proteins did coprecipitate (Fig. 6B), albeit to a much reduced extent in the case of the G121R mutation, which has been reported previously (34, 35). PF cells that exclusively expressed any of these mutant alleles, including G121R, had the same levels of editosome components as cells expressing Tet-regulatable WT KREPB5 or exclusively expressing WT KREPB5 from the β -tubulin locus (see Fig. S7B in the supplemental material). As expected, glycerol gradient analysis revealed that ~20S complexes were essentially absent in BF cells that exclusively expressed any mutant (Fig. 6C and E). In contrast, ~20S editosomes were present in PFs upon exclusive expression of all mutations except G121R (Fig. 6D and F).

Overall, our results demonstrate extensive BF-specific effects of KREPB5 mutations on *in vivo* editing and on editosomes and illustrate the phenomenon whereby specific amino acid changes within editosome proteins differentially affect cell growth, editosome integrity, and RNA editing in different life cycle stages (34). This work further reveals that this phenomenon is not limited to a few specific residues but is observed for many different residues throughout multiple domains of KREPB5. The results are consistent with the existence of intrinsic functional differences between BF and PF editosomes and with the involvement of KREPB5 in the processes that control differential editing between BF and PF life cycle stages.

DISCUSSION

Previous functional studies of *T. brucei* KREPB5 have been hampered by the lack of sequence conservation in identified protein motifs and by the absence of detectable homology to known motifs in large parts of the protein. Here, using random mutagenesis and a genetic complementation screen, we queried the *in vivo* function of 76% of the residues in KREPB5. We identified nine single substitutions that result in the loss of KREPB5 function in BF cells of *T. brucei*. Seven deleterious substitutions were found across

the degenerate RNase III domain, and two were found in the C-terminal region that has no detectable homology to known protein motifs. Previously described deleterious substitutions were also detected by our approach, thus illustrating the robustness of our screen. The lack of detrimental amino acid changes in the degenerate U1-like zinc finger or in the PUF motif is consistent with the findings of previous studies that showed that these motifs are not required for KREPB5 function in BF cells (34, 35) but could also be due to a lack of complete library coverage. The lethal substitutions that were identified resulted in inhibition of growth, inhibition of *in vivo* RNA editing, and a loss of 20S editosomes, thus identifying residues that are associated with stability, structural integrity, or the assembly of editosomes and editosome components in BF cells. We also identified 159 different amino acid substitutions in 134 residues that have no effect on the function of KREPB5 in BF cells. Indeed, KREPB5 variants with up to seven amino acid changes could complement the loss of KREPB5, showing that KREPB5 can tolerate a high number of nonconservative substitutions and yet still retain function. Together, these results delineate both deleterious and tolerated substitutions in KREPB5 that identify a novel functional domain and show that the degenerate RNase III motif is essential in *T. brucei*.

Our approach identified novel substitutions within the RNase III domain that severely inhibit KREPB5 function. The KREPB5 RNase III domain is degenerate, in that it lacks amino acids that are universally conserved in the active site of all known RNase III enzymes (35, 36). Mutation of the KREPB5 residues that align with catalytic RNase III residues had no effect on editing or *in vitro* cleavage of ESs, suggesting that the KREPB5 RNase III domain is noncatalytic (35). Although all characterized RNase III domains function as dimers (49, 50), the editing RNase III KREN1, KREN2, and KREN3 endonucleases are present as a single copy per editosome (26). We have therefore hypothesized that catalytic KREN1, KREN2, or KREN3 forms a heterodimeric RNase III with noncatalytic KREPB4 or KREPB5, to permit cleavage of mRNA at the ES. An analogous RNase III mechanism in which an artificial bacterial RNase III heterodimer with a single functional catalytic site can nick dsRNA has been described (51, 52). Seven deleterious substitutions that result in inhibition of editing and a loss of editosomes were found in the degenerate RNase III domain of KREPB5. These substitutions have the same effects on editing and editosomes as the loss of KREPB5 in BF cells (33), emphasizing the importance of this domain for KREPB5 function *in vivo* and highlighting a critical role for this domain in maintaining the structural integrity of editosomes. Homology modeling of KREPB5 with the template *Aquifex aeolicus* RNase III places G121 in the α -helical region that is important for dimerization of RNase III domains and that creates the functional catalytic center in other RNase III endonucleases (34, 49, 50). Mutation of this glycine to arginine in KREPB5 is predicted to result in steric interference that prevents normal dimerization. The F114S and L134P substitu-

FIG 5 Effects of KREPB5 mutations on *in vivo* RNA editing in BF and PFs. RT-qPCR analysis showing the abundances of RNAs from BF and PF KREPB5 CN cells in which the Tet-regulatable WT KREPB5 allele was repressed for 3 (BFs) or 4 (PFs) days (i.e., cells that exclusively expressed WT or mutant alleles from the β -tubulin locus) relative to those from cells that express the Tet-regulatable WT allele (Fig. 2 and 4). RNAs were reverse transcribed, preamplified, and analyzed using the Fluidigm BioMark HD multiplex qPCR system, and their levels of expression were normalized to the level of expression of telomerase reverse transcriptase (TERT) RNA, which was used as an internal control (see Materials and Methods for details). The relative abundances of RNAs from the KREPB5 alleles, preedited and edited RNAs, and the never-edited COI and ND4 mRNAs were determined in quadruplicate. (A) Heat map showing the log₁₀-transformed relative abundances of RNAs from BF cells (left) and PF cells (right), as indicated by the scale bar (bottom). (B) Selected examples of data from panel A showing the relative abundance on a log₁₀ scale for BF cells (left) and PF cells (right) that exclusively expressed WT KREPB5 or the L134P or V152E KREPB5 mutant allele.

FIG 6 Effects of KREPB5 mutations on BF and PF editosomes. BF and PF cells that exclusively expressed V5-tagged WT or mutant KREPB5 due to repression of the Tet-regulatable WT KREPB5 allele for 3 days or 4 days, respectively, were analyzed by Western blotting. (A and B) Anti-V5 immunoprecipitates from BF cells (12.5%) (A) and PF cells (6.25%) (B) probed with monoclonal antibodies against editosome proteins KREPA1, KREPA2, KREL1, and KREPA3 and anti-V5 antibody. Lysates from KREPB5 CN cells that had no tags and mock immunoprecipitations without antibody (not shown) were used as negative controls. (C and D) Glycerol gradient fraction 9 (~20S) from BF cells (C) and PF cells (D) probed as described for panels A and B. *, antibody binding to the protein A portion of the Tet-regulatable WT KREPB5-TAP protein in PF cells. (E and F) Glycerol gradient fractions from BF cells (E) or PF cells (F) expressing WT KREPB5 or the L134P or V152E KREPB5 mutant allele. IP, immunoprecipitation; WB, Western blotting.

tions also lie in this region and drastically change the properties of the encoded amino acid, a finding which is consistent with the effects on editosome structure and which supports the RNase III dimerization hypothesis. Further analyses are needed to determine whether KREPB5 can form heterodimeric structures with other RNase III domain-containing editosome proteins. The remaining four substitutions would not appear to directly affect RNase III dimerization in the homology model. V152 is structurally close to residues that correspond to the catalytic center and that coordinate the essential divalent metal ion cofactor in other RNase III endonucleases. It is possible that the V152E substitution alters the RNase III domain structure or interactions, producing subsequent effects on editosome structure. R77, V93, and S97 are placed away from the RNase III active site or the region responsible for dimerization, suggesting an interaction with or proximity to other proteins, consistent with the disruption of the integrity of the editosome upon substitution of these residues. Indeed, mass spectrometric analysis of cross-linked purified editosomes (S. M. McDermott, J. Luo, J. Ranish, and K. Stuart, unpublished data) reveals that lysines within 10 residues on either side of R77 cross-linked to the editosome proteins KREPA2, KREPA3, and KREPA6, while lysines within 10 residues on either side of V93 and S97 cross-linked to KREPB4 and KREPA2. A previous yeast two-hybrid analysis also showed that a region of KREPB5 that encompasses the RNase III domain (residues 40 to 253) can form a complex with KREPA3. These data are consistent with a key role for the RNase III domain in the interaction of KREPB5 with other editosome proteins and in maintaining the structural integrity of editosomes.

Two lethal single substitutions were also found in the C-terminal region of KREPB5. These resulted in inhibition of RNA editing and a loss of editosome complexes in BFs. Both the W258G and S320P substitutions resulted in severe inhibition of growth, inhibition of *in vivo* RNA editing, and a loss of 20S editosome components and complexes in BFs. These substitutions affect residues that lie within a region of KREPB5 that has no detected homology to known motifs or any characterized function. The effects of these substitutions show that this region is also associated with stability, structural integrity, or the assembly of editosomes and editosome components in BFs. The results of mass spectrometric analysis of cross-linked purified editosomes (McDermott et al., unpublished) are consistent with these results and reveal that residues within the C-terminal region cross-link with a number of other editosome proteins, including KREPA2, KREPA3, KREPA6, and KREPB4.

The ability to discover a function associated with the C-terminal region illustrates the utility of our approach. This approach also isolated a number of both lethal and tolerated substitutions in residues whose function would not have been predicted had we relied solely on the correlation between functional importance and sequence conservation (46). Comparative sequence analysis was therefore of limited value in predicting the position and function of residues critical for KREPB5. The divergence of *T. brucei* from other eukaryotes means that this will also be true for other essential *T. brucei* proteins. In contrast to sequence analysis and site-directed mutagenesis, our combination of random mutagenesis and genetic screening allowed the efficient generation and *in vivo* testing of many different amino acid substitutions without

any suppositions on the basis of the protein sequence. Our screening methodology is widely applicable to the functional investigation of proteins essential in *T. brucei* and other systems. The number of different protein variants that can be assayed in a single screen is limited only by the allele library size and the transfection efficiency of the mutant allele library (53). Strategies to increase transfection efficiency (54) would allow the generation of larger mutant libraries that could also be screened in an alternative high-throughput fashion, using deep sequencing, analogous to that used to screen previously described genome-wide RNA interference libraries in *T. brucei* (55, 56). The methodology can also be adapted to probe more specific questions. For example, it could be used to screen known *T. brucei* drug targets for substitutions that can lead to drug resistance (57) or screen proteins for substitutions that abolish essential interactions with partners. Indeed, random mutagenesis has previously been used in combination with yeast reverse two-hybrid screening for the isolation of edgetic alleles and the evaluation of the *in vivo* roles of individual interactions (37, 58).

Of the nine deleterious substitutions that we describe in the KREPB5 RNase III domain and C-terminal region, eight had BF-specific effects on growth, editing, and editosomes that were not observed in PFs. Only the G121R substitution affected editing and editosomes in both PFs and BFs, underscoring the significance of this residue and therefore, potentially, RNase III dimerization for KREPB5 function in both life cycle stages (34, 35). Site-directed single amino acid changes in the RNase III domain and PUF motif in KREPB5 that differentially affect cell growth, editosome integrity, and RNA editing in BF and PF cells have previously been characterized (34). This work reveals that this phenomenon is not restricted to a few specific residues in KREPB5 but is extensive and observed for many different residues throughout multiple domains of KREPB5. The results support a role for KREPB5 in the processes that differentially regulate editing between life cycle stages and are consistent with the existence of inherent functional differences between BF and PF editosomes. We hypothesized that these differences and the differential effects of single amino acid substitutions on protein function could be due to differences in the editosome protein conformation and/or posttranslational modifications between life cycle stages and their consequent effects on stability, assembly, or interactions among proteins within the editosomes or with other complexes (34). The results of this study further suggest that the explanation for this phenomenon is not restricted only, for example, to the differential posttranslational modification of KREPB5, as different types of residues are altered by different substitutions.

Altogether, we present a robust, generally applicable *in vivo* method that we used to analyze hundreds of KREPB5 variants in parallel and to reveal amino acid residues that are important for protein function. We show that the degenerate RNase III domain is essential for function in *T. brucei*, identify important residues in an uncharacterized C-terminal region with no homology to known motifs, and characterize a wide range of mutations that provide powerful support for a role of KREPB5 in the differential regulation of RNA editing. This is a useful approach for investigation of proteins in an early-branching, divergent eukaryote such as *T. brucei*, where sequence homology has been of limited value in predicting the position and function of critical residues.

ACKNOWLEDGMENTS

We declare that we have no conflicts of interest with the contents of this article.

We thank Atashi Anupama for assistance with sequence analysis and members of the K. Stuart lab for helpful discussion.

This work was supported by National Institutes of Health grant R01 AI014102 to K.S.

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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