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KREPA4, an RNA binding protein essential for editosome integrity and survival of *Trypanosoma brucei*

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ABSTRACT

The 20S editosome, a multiprotein complex, catalyzes the editing of most mitochondrial mRNAs in trypanosomatids by uridylyl insertion and deletion. RNAi mediated inactivation of expression of KREPA4 (previously TbMP24), a component of the 20S editosome, in procyclic form *Trypanosoma brucei* resulted in inhibition of cell growth, loss of RNA editing, and disappearance of 20S editosomes. Levels of MRP1 and REAP-1 proteins, which may have roles in editing but are not editosome components, were unaffected. Tagged KREPA4 protein is incorporated into 20S editosomes in vivo with no preference for either insertion or deletion subcomplexes. Consistent with its S1-like motif, recombinant KREPA4 protein binds synthetic gRNA with a preference for the 3' oligo (U) tail. These data suggest that KREPA4 is an RNA binding protein that may be specific for the gRNA Utail and also is important for 20S editosome stability.

Keywords: 20S editosome; RNA binding; RNA editing; editosome integrity

INTRODUCTION

Mitochondrial (mt) mRNAs in trypanosomes undergo RNA editing, which inserts and deletes uridylylates (Us) to produce mature functional mRNAs (Madison-Antenucci et al. 2002; Simpson et al. 2004; Stuart et al. 2005). The number of Us that are inserted and deleted and the sites of their insertion and deletion are specified by guide RNAs (gRNAs). RNA editing occurs by multiple cycles of four key catalytic steps, endonucleolytic cleavage of the pre-mRNA followed by the addition or removal of one or more Us at the 3' end of the 5' cleavage fragment for insertion or deletion editing, respectively, and the ligation of this processed 5' RNA fragment with the 3' RNA fragment. Kinoplastid RNA editing (KRE) proteins occur as pairs or sets of related proteins in multiple complexes. The enzymes for editing are contained within multiprotein complexes, 20S editosomes, that can accurately edit single sites in vitro. 20S editosomes contain at least 20 proteins whose specific functions are being elucidated. KREL1 and KREL2 are deletion and insertion mRNA ligases, respectively (Huang

et al. 2001; Cruz-Reyes et al. 2002; Drozd et al. 2002; Schnauffer et al. 2003), KRET2 is an mRNA terminal uridylyl transferase (TUTase) (Ernst et al. 2003), KREX1 and KREX2 are U-specific exonucleases (exoUases) (Kang et al. 2005; N.L. Ernst, B. Panicucci, J. Carnes, and K. Stuart, unpubl.), KREN1 and KREN2 are deletion and insertion endonucleases, respectively (Carnes et al. 2005; Trotter et al. 2005), and KREH1 is an RNA helicase (Missel et al. 1997). The six 20S editosome KREPA proteins all have C-terminal OB-fold-like motifs, and the eight KREPB proteins all have a U1-like zinc finger motif (Schnauffer et al. 2003; Worthey et al. 2003). Their potential functions are described below. Other complexes and proteins function in RNA editing and may interact with 20S editosomes. These include the KRET1 complex that adds the gRNA 3'oligo(U) tails, the MRP1 and two proteins that can anneal gRNA and mRNA, and a complex with RBP16 that affects the abundance of edited and unedited mRNAs (Koller et al. 1997; Pelletier et al. 2000; Müller et al. 2001; Aphasizhev et al. 2003b; Pelletier and Read 2003; Vondruskova et al. 2005). Additional proteins such as TbRGG1 and REAP1 may function in RNA editing or its regulation (Madison-Antenucci et al. 1998; Vanhamme et al. 1998; Madison-Antenucci and Hajduk 2001).

The 20S editosomes are physically and functionally organized into subcomplexes. Heterotrimeric KRET2, KREL2, and KREPA1 insertion subcomplexes and KREX2,

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KREL1, and KREPA2 deletion subcomplexes are evident from a variety of in vitro and in vivo studies (Cruz-Reyes et al. 2002; Huang et al. 2002; Aphasizhev et al. 2003a; Ernst et al. 2003; Schnauffer et al. 2003). KREPA1 and KREPA2, which both have two C2H2 zinc fingers, have been suggested to coordinate the order in which the catalytic steps occur during insertion and deletion editing, respectively (Schnauffer et al. 2003; Law et al. 2005). A recent report suggests that the 20S editosome protein KREPA3 contributes to both endo- and exoribonuclease activities of RNA editing (Brecht et al. 2005). KREPA4, which is the focus of this article, was previously designated TbMP24 based on its preprocessed molecular weight of 23.7 kDa and was identified by mass spectrometric analyses of editosomes that were biochemically purified or immunoprecipitated by a monoclonal antibody (MAb) specific for editosome protein KREPA2 (Panigrahi et al. 2003). KREPA4 belongs to a family of six proteins (KREPA1–6) that have a conserved C-terminal region with an OB-fold-like domain, the three largest of which also have C2H2 zinc fingers (Panigrahi et al. 2001b; Schnauffer et al. 2003; Worthey et al. 2003). The KREPA4 gene is immediately upstream of the KREPA6 gene in all three trypanosomatid genomes, suggesting that they are the result of a gene duplication event (Worthey et al. 2003). The functions of these proteins are unknown. KREPB1–8 all have U1-like zinc finger motifs and KREPB1–5 all also have RNase III-like motifs, although this motif in KREPB4 and -5 diverges substantially from the canonical RNase III motif (Panigrahi et al. 2003; Worthey et al. 2003). KREPB1–3 also have putative double-stranded RNA binding motifs and have endonuclease activity (Worthey et al. 2003; Carnes et al. 2005; Trotter et al. 2005). Otherwise, the functions of the KREPB proteins are unknown, except that KREPB5 is essential to the editosome integrity (Wang et al. 2003).

Some information is emerging on which proteins are essential for editosome structural integrity. RNAi inactivation of KREPA1 expression has been shown to preferentially inhibit insertion editing and result in the loss of adenylation editing ligase KREL2 and to shift editosomes to ~15S (Drozd et al. 2002; O'Hearn et al. 2003). Similarly, inactivation of KREPA2 leads to substantial disruption of the 20S editosome and loss of editing ligase KREL1 (Huang et al. 2002). The effect of down-regulation of KREX1 expression results in a progressive decrease in the S value of the 20S editosome over time and preferential reduction of in vitro precleaved U-deletion editing (Kang et al. 2005).

We report here genetic and functional studies that show that KREPA4 is essential for RNA editing and 20S editosome structural integrity. Tagged KREPA4 cosediments at ~20S on glycerol gradients with the four key enzyme activities that catalyze in vitro editing. RNAi-mediated inactivation of KREPA4 in vivo results in editosome disintegration, with consequent loss of editing and cell growth. Comparative in silico analyses predict that

KREPA4 has an N-terminal region of low compositional complexity and a midregion with a potential S1 motif. Recombinant KREPA4 protein binds synthetic gRNA that contains a U tail with specificity for oligo (U). These data suggest that KREPA4 may function in protein–protein interactions and interact with RNA, potentially the 3' U tail of gRNA.

RESULTS

KREPA4 is essential for trypanosome growth and RNA editing

The role of KREPA4 in editing was explored by RNAi mediated repression (knockdown) of its gene expression by generating double-stranded RNA (dsRNA) against the entire KREPA4 coding region. The dsRNA was expressed using a construct with opposing tetracycline (tet)-inducible T7 RNA polymerase promoters and stably integrating it into the nontranscribed spacer of the rDNA locus (Wang et al. 2000). This was done in procyclic form *Trypanosoma brucei* (PF) strain 29–13 (Wirtz et al. 1999), which contains T7 RNA polymerase and tet repressor genes so that dsRNA is induced by addition of tet. Knockdown of KREPA4 mRNA expression and growth inhibition was observed upon expression of the dsRNA (Fig. 1A). Constructs with the 5' 315 bp including 60 bp of the 5' UTR or the 3' 327 bp of KREPA4 did not inhibit growth despite some reduction in its mRNA abundance (data not shown). Northern analysis of RNA from the full-length KREPA4 RNAi cell lines with an oligonucleotide probe that is complementary to the KREPA4 5' UTR region and can discriminate the dsRNA from the target mRNA showed that KREPA4 mRNA was reduced in the cells in which full-length RNAi was induced, relative to the uninduced cell lines (days 2 and 4 –RNAi lanes).

Expression of the full-length KREPA4 dsRNA dramatically inhibited cell proliferation (Fig. 1A). This along with the mRNA knockdown suggests that the KREPA4 protein expression is repressed (down-regulated) upon induction of the full-length dsRNA, although we could not assess this directly due to the lack of antibody that is specific for KREPA4. Repression of KREPA4 protein expression is also suggested by the absence of other editosome proteins as described below. Cell growth essentially ceased after 6 d of growth in the presence of tet and resumed by day 19, possibly due to loss of RNAi knockdown (Chen et al. 2003). RNA editing in vivo was significantly reduced after KREPA4 repression by RNAi induction for 6 d as shown by real-time PCR analysis (Fig. 1B). The levels of edited A6, RPS12, COIII, CYb, and COII mRNAs were reduced by ~60%–80% while the levels of the pre-edited transcripts were essentially unchanged or somewhat increased (CYb and COII). The levels of COI and ND4 mRNAs that do not

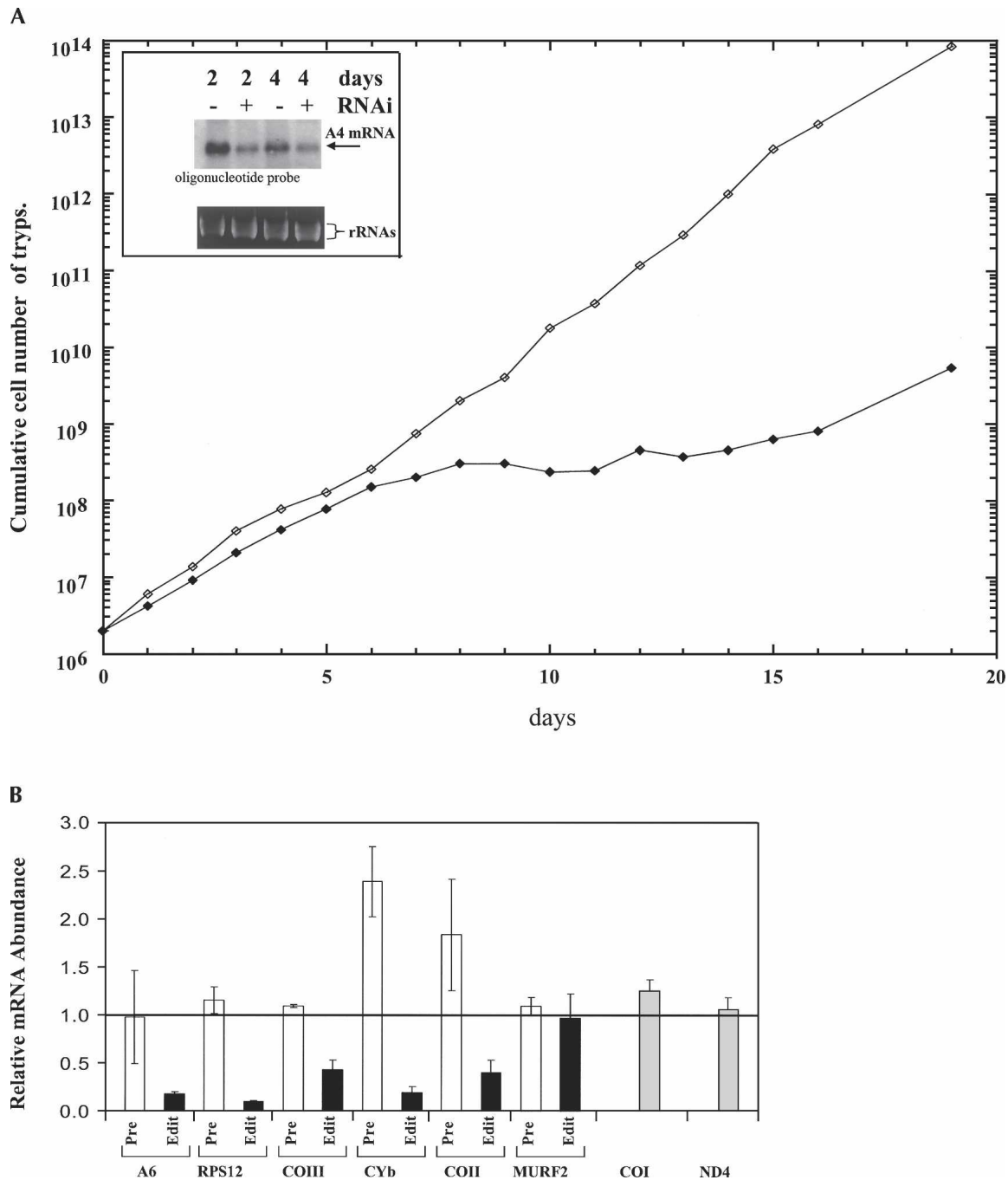


FIGURE 1. Down-regulation of KREPA4 expression affects trypanosome growth and edited RNA abundance. (A) Growth curve of cells in which KREPA4 is expressed in the absence of RNAi induction (open diamonds) or repressed after RNAi induction with 1 μ g/mL tet (solid diamonds). The cumulative cell numbers reflect the normalization of the cell densities by the dilution factor. (Inset) Northern blot of total RNA hybridized with an oligonucleotide probe complementary to the 5' UTR region of KREPA4. RNA was collected on the indicated days from induced (+RNAi) or uninduced cells (–RNAi). Ribosomal RNA is shown as a loading control. (B) KREPA4 repression reduces RNA editing in vivo. Real-time PCR analysis of RNA from KREPA4 RNAi cells in the absence or presence of RNAi induction for 6 d. The abundance of pre-edited (pre, white bars) and edited (edit, black bars) mRNAs from repressed cells is shown relative to that from cells in which KREPA4 is expressed. The relative abundance of never edited COI and ND4 mRNAs (medium gray bars) are also shown. The RNA levels were normalized to 18S rRNA. The thick gray line at 1 indicates no relative change in mRNA level, with anything above or below this line representing an increase or decrease in mRNA levels, respectively. Error bars represent the standard deviation from at least three replicates.

undergo editing were unaffected. The level of edited MURFII mRNA did not decrease, possibly reflecting greater stability. Northern analysis showed that KREPA4 mRNA levels were reduced upon RNAi induction although real-time PCR using a primer set for the 3' UTR did not show a corresponding reduction (data not shown) perhaps because this region of the RNA was not targeted by the RNAi. In addition, real-time PCR showed that the mRNA level of KREPA6, the editosome protein most closely related to KREPA4, was not changed following KREPA4 RNAi knock-down (data not shown). Thus, knockdown of KREPA4 expression substantially inhibited both growth and editing *in vivo*, confirming its role in editing.

Inactivation of KREPA4 expression results in loss of ~20S editosomes

Western analyses of glycerol gradient fractions from whole-cell lysates with MAbs specific for four editosome proteins revealed a dramatic reduction in editosome proteins after 4 d of growth in the presence of tet. The KREPA1, KREPA2, KREPA3, KREPA6, and KREL1 proteins that cosediment at ~20S in cells grown in the absence of tet were dramatically reduced in abundance following the repression of KREPA4 expression (Fig. 2A). Some KREL1, KREPA2, and to lesser extent KREPA1 and KREPA3 remained but shifted to a higher region of the gradient. Thus, the loss of editing is paralleled by the loss of editosomal proteins and of ~20S editosomes. These data indicate that KREPA4 is important to editosome structural integrity. Western analyses of total cell lysates from these studies were not sufficiently sensitive to directly determine the overall abundance of the editosome proteins (data not shown). Western analyses of glycerol gradient fractions from whole-cell lysates with MAbs specific for other RNA binding proteins that may be involved in editing but are not integral components of the editosome including MRP1 (previously gBP21) and REAP-1, showed no difference in protein levels or sedimentation between cells in which KREPA4 was expressed or repressed (Fig. 2B,C). The slightly greater signal intensity of samples when KREPA4 was repressed appears to be due to loading more protein on these gradients, as indicated by the greater signal in the PGK loading control, a protein that is not involved in editing (Fig. 2D). These data indicate

that the role of KREPA4 is restricted to the ~20S editosome.

Repression of KREPA4 expression also resulted in the loss of the *in vitro* RNA editing activities that normally sediment at ~20S. Precleaved insertion editing, which entails linked TUTase and ligase activities, and precleaved deletion editing, which entails linked exoUase and ligase activities, were greatly diminished in the glycerol gradient 20S fractions (centered around fraction 13) from whole-cell lysates after 4 d of RNAi induction (Fig. 3A,B, lanes labeled KREPA4 repressed). Western analyses and activity profiles however were not identical since the relative abundance of non-editosome proteins in these complex mixtures as well as proteins that affect activity (e.g., ligases) could differentially affect Western versus activity signals. Nevertheless, traces of ligated products of both the precleaved insertion and deletion substrates were detected, as was substantial exonuclease activity. The former may reflect residual editosome ligase activity (see above), while the latter probably reflects contaminating nucleases from the whole cell lysates that are not U specific and result primarily in -3U products as shown previously (Wang et al. 2003). Thus, the partial loss of the catalytic activities associated

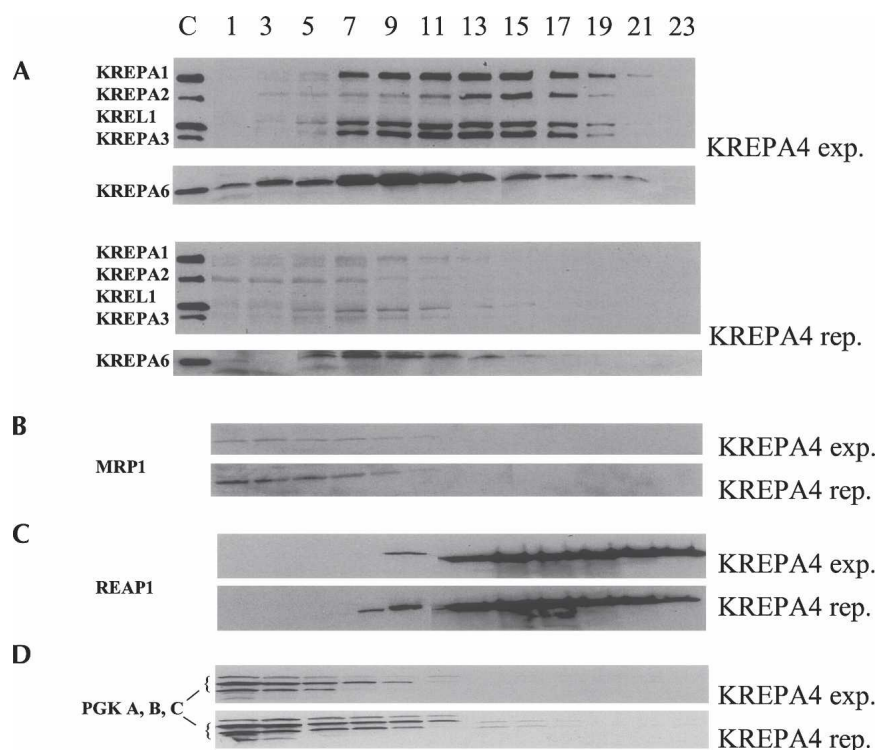


FIGURE 2. Loss of the 20S editosome upon down-regulation of KREPA4 expression. Western analysis of glycerol gradient fractions or a partially purified editosome fraction (control, c) with the KREPA1, -2, -3, -6, KREL1 (A), MRP1 (B), REAP1 (C), and PGK (D) antibodies. Lysates of equivalent numbers of cells (3×10^9) and protein in which KREPA4 was expressed (exp.) or repressed (rep.) by RNAi induction for 4 d were subjected to glycerol gradient fractionation followed by Western blot analysis. The PGK isoforms A, B, and C were used as a loading control.

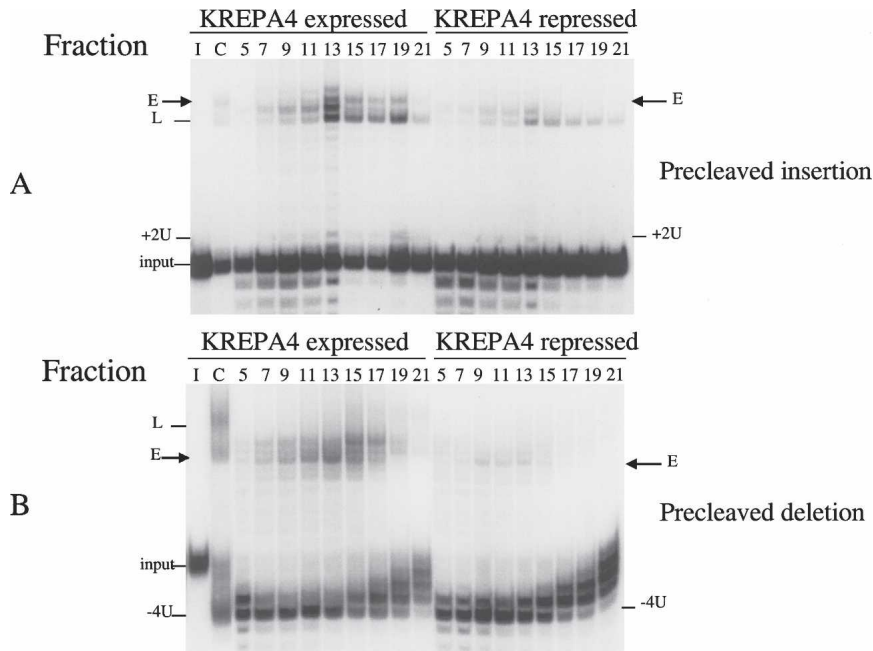


FIGURE 3. Reduction of RNA editing catalytic activities *in vitro* upon KREPA4 repression. Lysates of cells in which KREPA4 was expressed or repressed by RNAi induction for 4 d were fractionated on glycerol gradients for 5 h and the fractions were assayed for editing activities. (A) Precleaved U insertion RNA editing. Input RNA with two added Us (+2U), edited RNA with the insertion two Us (E), or the ligated product of the 5' and 3' RNA fragments without any added Us (L) are indicated. (B) Precleaved U deletion editing. Input RNA with four Us removed (−4U), RNA edited by the deletion of 4 Us (E), and ligated RNAs without any Us removed (L) are indicated.

with RNA editing confirms the involvement of KREPA4 in editing.

Physical and functional association of KREPA4 with the editosome proteins

The structural association of KREPA4 protein with other editosome components was assessed using a cell line containing a tet-inducible version of the KREPA4 gene fused to a C-terminal TAP tag (Rigaut et al. 1999). Whole-cell lysates of the uninduced and induced cells were fractionated by glycerol gradient fractionation for 5 h (Fig. 4A–D). Western analysis of the glycerol gradient fractions with a mixture of four MAbs that are specific for 20S editosome proteins revealed increased signal at the position expected for tagged KREPA4 in the 20S (centered at fraction 11) and less than 20S region of the gradient in induced compared to uninduced cells (Fig. 4A,B). This increased signal is likely due to the tagged KREPA4, which comigrates with KREPA3, since both the primary and secondary antibodies can bind the Protein A moiety of the tagged KREPA4 protein. The stronger signal in fractions 3 and 5 from induced cells with MAbs implies that the tagged KREPA4 was present throughout the gradient with some of the tagged protein at or near the top, which could have led to its degradation in the absence of its interacting

partner(s). To distinguish the KREPA3 signal from tagged KREPA4, glycerol gradient fractions were probed with a mixture of KREPA1 and KREL1 MAbs. Tagged KREPA4 was only detected in cells that were induced with tet (Fig. 4C,D). Overall these data suggest that some of the tagged KREPA4 is in ~20S editosomes, as further implied below.

Tagged editosomes were purified from induced KREPA4 TAP-tagged cells by sequential IgG-sepharose affinity chromatography followed by TEV protease elution, glycerol gradient sedimentation, and calmodulin resin affinity chromatography as described previously (Schnauffer et al. 2003). The TEV eluate from the IgG sepharose contained complexes with the KREPA1, KREPA2, KREPA3, and KREL1 proteins that cosedimented in gradients (Fig. 4E). The reduced signal in the KREPA3 region compared to Figure 4B is due to removal of the Protein A portion of the tag by TEV protease digestion, further implying integration of the tagged protein into 20S editosomes. This is also suggested by the

presence of the KREPA1, KREPA2, KREPA3, and KREL1 proteins in pooled fractions 11–14 that were further purified by calmodulin affinity chromatography (Fig. 4F). Some KREL1 and KREPA2 were detected with the MAbs and by adenylation in fraction 5 (Fig. 4E). None of the KREPA1, KREPA2, KREPA3, or KREL1 proteins were found in pooled fractions 3–6 after calmodulin affinity chromatography (Fig. 4F). Perhaps the abundance of these proteins was too low for detection (possibly due to proteolysis) or they are loosely associated with tagged KREPA4 in these fractions and did not remain associated with the tagged KREPA4 during the final purification step. Taken together these results indicate that KREPA4 is an integral component of 20S editosomes but that it does not appear to be a stable component of smaller subcomplexes.

We assayed the pooled glycerol gradient fractions (fractions 3–6 and 11–14) before and after calmodulin affinity purification for precleaved insertion and deletion editing. Pooled fractions 11–14 catalyzed both precleaved insertion and deletion before and after they were subjected to calmodulin purification and thus contain the exoUase, TUTase, and ligase editing activities (Fig. 4G). These activities were only detected in pooled fractions 3–6 prior to calmodulin purification, which is consistent with the lack of detectable editosome proteins in these fractions (Fig. 4F). On the other hand, pooled fractions 11–14 contain the exoUase/TUTase

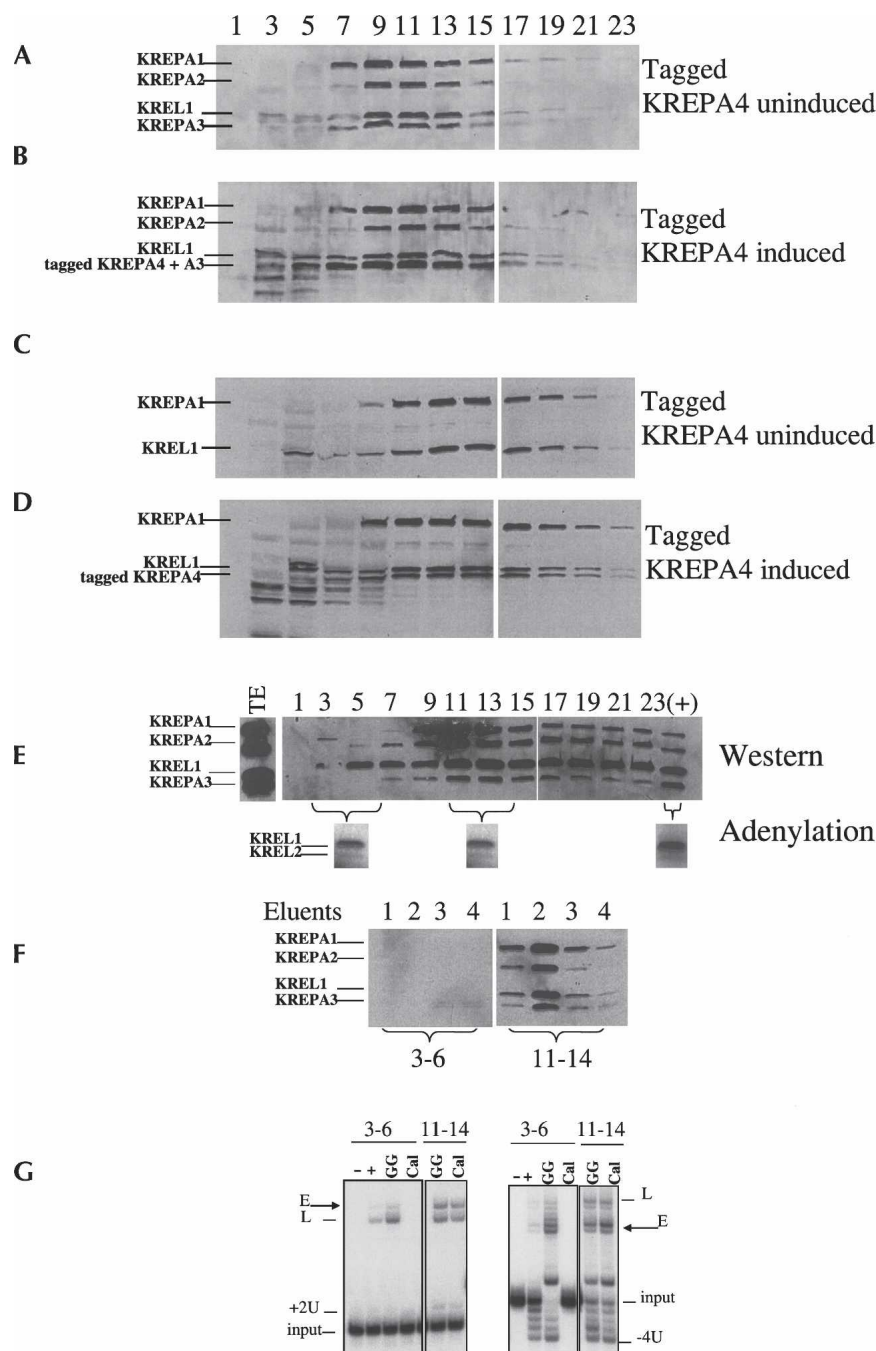


FIGURE 4. Characterization of proteins associated with TAP-tagged KREPA4 *in vivo*. Whole-cell extracts from equivalent numbers of cells (3×10^9) and protein in which expression of TAP-tagged KREPA4 was not induced (A,C) or induced with $1 \mu\text{g/mL}$ tet for 2 d (B,D) were subjected to glycerol gradient fractionation followed by Western blot analysis with antibodies as indicated. (E) Proteins associated with tagged KREPA4 in induced cells were purified by IgG affinity chromatography and eluted with TEV protease. The TEV eluate was then separated on a 10%–30% glycerol gradient and subjected to Western analysis. (Lower panel) The pooled glycerol gradient fractions (3–4 or 11–14) were analyzed for the presence of the editing ligases by adenylation with $[\alpha\text{-}^{32}\text{P}]\text{ATP}$. The RNA editing ligases are indicated as KREL1 and KREL2. (+) Partially purified editosome fraction used in the Western and adenylation assays (Panigrahi et al. 2001a). (F) Pooled glycerol gradient fractions 3–6 or 11–14 were further purified by calmodulin affinity and the four eluates were subjected to Western analysis with the MABs indicated. (G) *In vitro* editing assays of the second eluate from the calmodulin purification step (cal) with precleaved insertion (left panel) and precleaved deletion (right panel) editing assays. The negative control (–) contains no extract and the positive control (+) has partially purified mitochondrial extract. Pooled glycerol gradient fractions 3–6 or 11–14 were assayed directly (GG) or after further purification by calmodulin affinity (cal). Editing intermediates and end products are indicated as in Figure 2.

and ligase editing activities. Overall, these results demonstrate the association of KREPA4 with 20S editosomes.

Compositional analysis and structural prediction of KREPA4

Structural prediction and compositional analysis of KREPA4 identified not only the amphipathic N terminus expected for a mt protein (Fig. 5A, underlined; data not shown) and the OB fold previously reported (Worthey et al. 2003) but also a putative S1 motif within the OB fold (amino acids 75–148, highlighted in yellow, and see below) and two low compositional complexity regions (amino acids 25–36 and 53–67, highlighted in green). In addition, KREPA4 contains 24 arginines and 24 glycines as the most abundant amino acids (11% by number for each amino acid). The presence of an arginine-rich sequence and a putative S1 motif strongly suggests that KREPA4 is an

RNA binding protein. Low complexity regions in proteins typically lack catalytic activities; however, they have been found to have important biological functions, most often associated with protein–protein interactions (Wootton and Federhen 1996). This sequence analysis thus suggests that KREPA4 has the capacity to interact with both the RNA and other proteins in the editosome.

The S1 motif is a type of OB fold, which is found in a large number of RNA-associated proteins. Motif scanning of the *T. brucei* KREPA4 protein (*Tb*KREPA4) using Profile-Scan (Pagni et al. 2004) detected a weak similarity match to a S1 motif (score of 6.7 with 10 being the highest score) within its midregion (amino acids 75–148). However, the *Trypanosoma cruzi* and *Leishmania major* KREPA4 orthologs (*Tc*KREPA4 and *Lm*KREPA4) are more diverged and do not appear to contain an S1 motif (see Discussion). A multiple sequence alignment of the potential *Tb*KREPA4 S1 motif (residues 75–148) with two S1 motifs containing

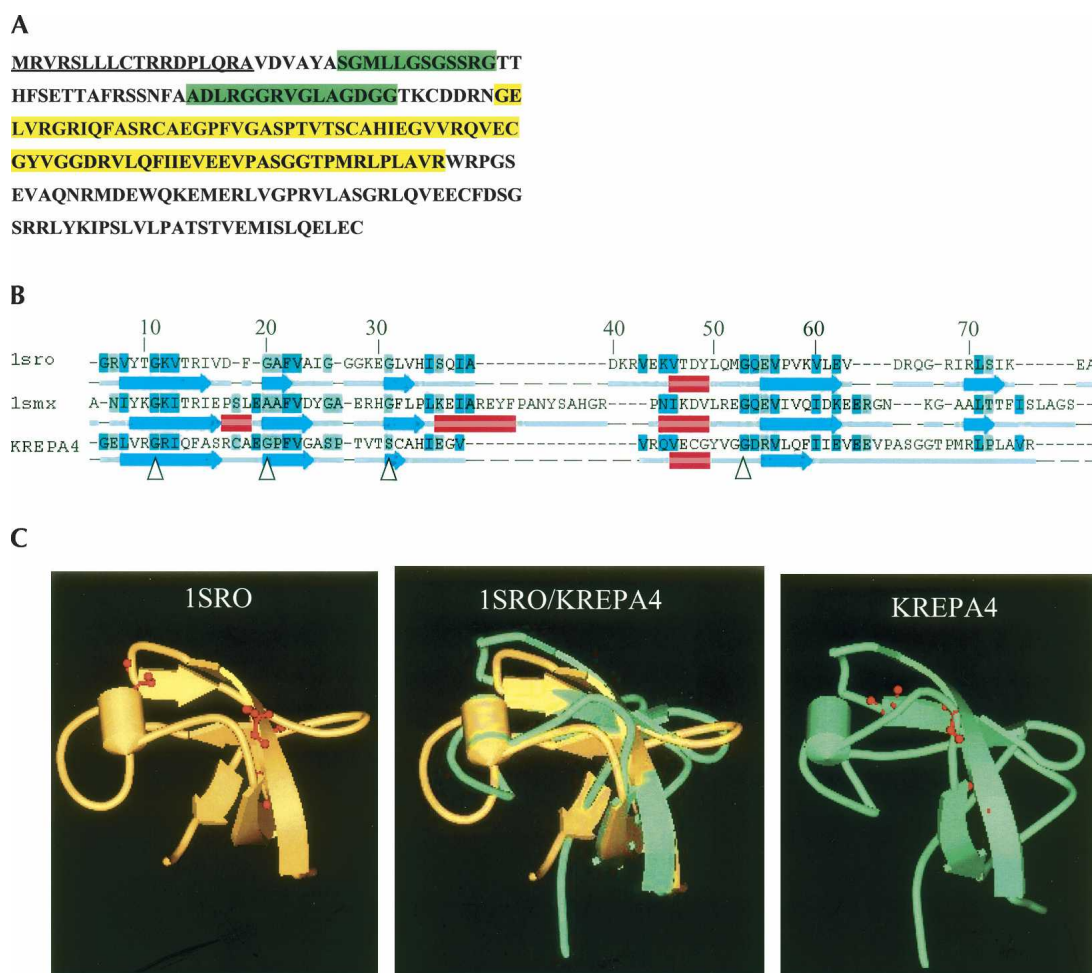


FIGURE 5. Amino acid sequence analysis and a structural model of the *T. brucei* KREPA4 S1 motif. (A) The putative 18-amino acid mitochondrial import presequence is underlined. The predicated low compositional complexity regions are highlighted in green and the putative S1 motif is in yellow. (B) A multiple sequence alignment of the S1 motifs in *E. coli* PNPase (1sro), RNase E (1smx), and KREPA4. Residues of 1sro and 1smx that are critical for the OB fold are indicated by triangles and are numbered as per *E. coli* 1sro. (C) Comparison of the 1sro structure (yellow), the KREPA4 model (green) superimposed with the 1sro structure (1sro/KREPA4), and the modeled KREPA4 structure (green). The side chains of glycine residues (red) that are important for the OB fold are shown as ball and stick.

proteins of *Escherichia coli* PNPase (1sro, residues 6–76) and RNase E (1smx, residues 10–96) that have known structures that were solved by NMR and X-ray crystallography, respectively, is shown in Figure 5B. The most conserved residues in S1 motif are four glycine residues (G11, G20, G31, and G53; numbered per *E. coli* 1sro) of which three are conserved in KREPA4 (G80, G91, G120; numbered per *T. brucei*) and the fourth is a conservatively replaced (S102). A valine that is occasionally replaced by isoleucine, as in KREPA4 (I82), and contributes to the hydrophobic core of the five-stranded antiparallel β barrel, is also well conserved. S1 motifs have a unique turn of a 3_{10} helix at the end of strand 3 that is not found in other OB-fold proteins, and the isoleucine and valine (I106 and V109) that could form this helix are conserved in KREPA4.

The three-dimensional (3-D) structure of *Tb*KREPA4 was predicted on the basis of this alignment and the known structures of the S1 motifs of 1sro and 1smx as templates. This homology modeling and superposition of the *Tb*KREPA4 and 1sro 3-D structures, with an average carbon distance of 1.7 Å, reveals the conserved structural core and many shared features of the S1 motif. Our modeling data suggest that they may have similar structures, with the conserved glycines distributed in the interior of the OB fold (Fig. 5C). These glycines are probably involved in structural organization similar to the superfamily of OB-fold proteins (Bycroft et al. 1997). The *Tb*KREPA4 model most notably lacks the fifth β -strand but it conserves features of strands 1 and 4 that contribute to homodimerization of the S1 fold in RNase E and aspects of strands 2 and 3 with their exposed residues that are the likely RNA binding region. Based on this structural model *Tb*KREPA4 can be predicted to contain an S1 RNA binding motif that may mediate RNA substrate binding specificity and also have the capacity to interact with other editosome proteins and hence bring the RNA and its catalysts into proximity.

KREPA4 purification and functional characterization

An inducible N-terminal His₆-KREPA4 fusion protein lacking the first 40 amino acids was expressed in *E. coli* and was soluble (Fig. 6A). Despite several attempts, we were unable to obtain sufficient soluble full-length recombinant protein or a version from which the N-terminal 18 amino acids were removed. While the purified protein lacked the predicted mitochondrial import signal and the first low compositional complexity region, it retained its second low compositional complexity region and the predicted S1 motif. Small amounts of the protein were expressed without induction, suggesting leaky expression of the T7 promoter (Fig. 6A, lane 1). The protein was purified via its His tag to near homogeneity (Fig. 6A, lanes 5,6; see Materials and Methods), and Western analysis using an anti-His antibody detected primarily protein of the expected size and a smaller amount with the apparent size of a

homodimer (Fig. 6A, lane 6). The homodimer signal, which represents a small portion of the overall KREPA4 signal, might be due to the incomplete denaturation of the protein due to the high concentration of the protein and the sensitivity of the Western blots, since the corresponding signal is absent in the gel stained with Coomassie (see lane 5). In addition, this signal was only detected when the cells were induced (data not shown). Finally, this higher molecular weight band was also detected on Western blots when a larger fragment of KREPA4 was expressed in *E. coli*, and the signal for the potential homodimer band was correspondingly larger (data not shown).

Purified KREPA4 protein was assayed for RNA editing-associated catalytic activities and RNA binding. Enzymatic assays for the known catalytic activities in RNA editing using in vitro RNA editing substrates did not detect any of the four major catalytic activities of editing, endoribonuclease, TUTase, exonuclease, or RNA ligase (data not shown).

Gel mobility shift assays using radiolabeled gA6[14] guide RNA that has an oligo (U) tail were performed in the presence of increasing amounts of unlabeled competitors (Fig. 6B). Incubation of recombinant KREPA4 with gA6[14] RNA resulted in a shifted product in the absence of competitor. Quantification of gel shift signals was normalized against the lanes where the competitor RNA was absent. The oligo (U) tail region of the gRNA molecule appears to be a major determinant for binding of the protein. Formation of the shifted product was specific for the gRNA with the U tail since its formation was inhibited by ~75% by addition of 100-fold molar excess of unlabeled homologous RNA but not heterologous Bluescript RNA (Fig. 6B). Furthermore, KREPA4 resulted in gel shifts of labeled gRNA without a U tail in a nonspecific manner, since this binding was unaffected in the presence of 100-fold molar excess of unlabeled homologous RNA (data not shown). Consistent with this data, competition with a 100-fold molar excess of gA6[14] gRNA devoid of the oligo (U) tail or A6 pre-mRNA mRNA inhibited product formation by only ~10%. However, addition of an equimolar amount of poly (U) inhibited product formation by 35% while 10- or 100-fold molar excess of poly (U) inhibited product formation by ~80%. Radiolabeling of the poly U indicated that it had a size range of 10–70 nt (data not shown). This may partially explain why poly (U) is a more efficient competitor than the guide RNA with an average oligo (U) tail length of 10–15 nt.

In contrast, up to a 100-fold molar excess of poly (G), poly (C), or poly (A) inhibited product formation by a maximum of 23%. Thus KREPA4 specifically binds the poly (U) tail of gA614 gRNA.

DISCUSSION

This study shows that KREPA4 is an essential protein component of the ~20S RNA editing complex of *T. brucei*.

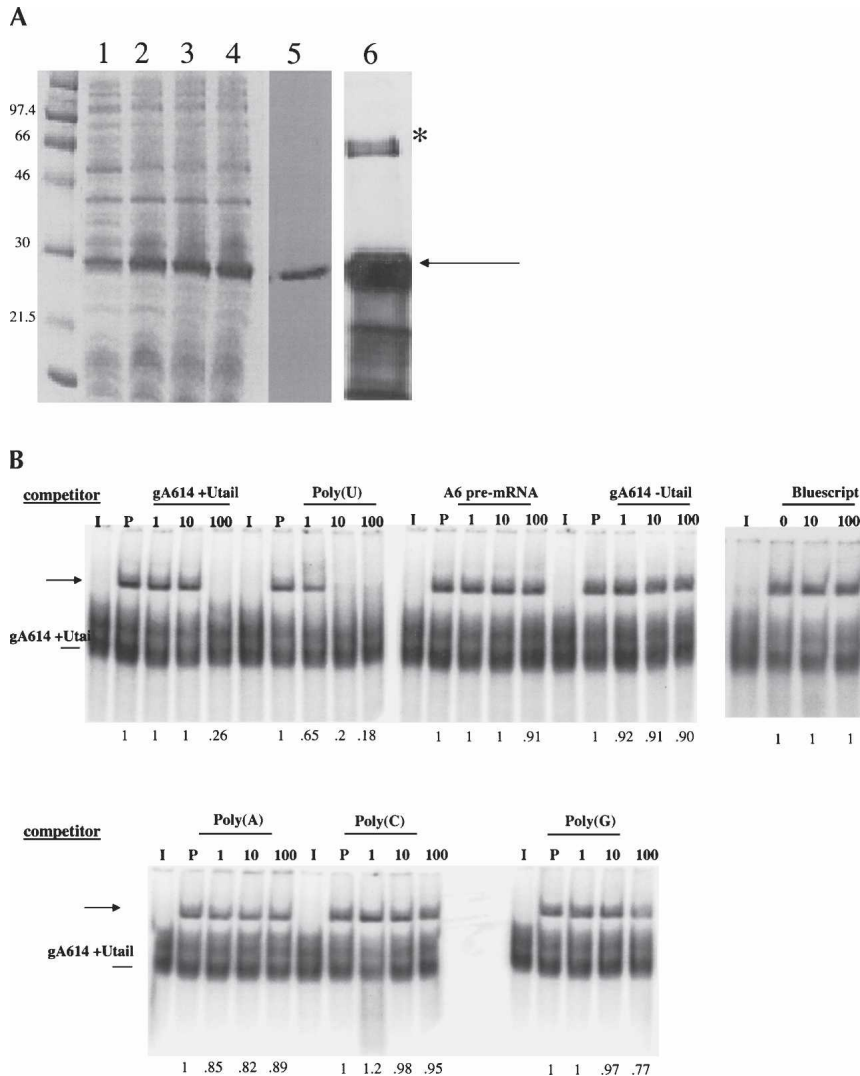


FIGURE 6. Expression and RNA binding characteristics of recombinant KREPA4. (A) Recombinant KREPA4 lacking the first 40 amino acids was induced in *E. coli* with 1 mM IPTG at time point 0. Proteins from cells at time 0 (lane 1), 1 h (lane 2), 2 h (lane 3), and 3 h (lane 4) post-induction were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Purified recombinant KREPA4 was separated by SDS-PAGE and stained with Coomassie Brilliant Blue (lane 5). Western blot of purified recombinant A4 with a monoclonal antibody against the His-tag (lane 6). The arrow indicates the position of recombinant KREPA4, and the asterisk represents a potential recombinant KREPA4 dimer. Size markers in kilodaltons are shown on the left. (B) Recombinant KREPA4 (20 ng, 800 fmol) was incubated with 1 fmol of radiolabeled gA6[14] with a U tail in the presence of varying amounts of competitor RNAs. I indicates the radiolabeled input RNA, P, the radiolabeled RNA in the presence of KREPA4 without competitor RNA, and the numbers above the panels indicate the fold excess of unlabeled competitor RNAs. RNA-protein complexes were resolved by electrophoresis on 9% nondenaturing acrylamide gels and visualized with a PhosphorImager. The arrows indicate the gel shifts of radiolabeled RNA with KREPA4, and the numbers below the panels indicate percent of shift normalized to the shifts in the absence of competitor (P).

Regulated loss of KREPA4 results in a dramatic reduction in the growth of PF trypanosomes, loss of editing in vivo and the in vitro editing-associated catalytic activities, as well as loss of editosomes. We therefore conclude that KREPA4 is essential to the stability and perhaps structure of the editosome. KREPA4 contains a predicted S1 motif

and the recombinant protein binds to synthetic gRNA, specifically the gRNA 3'-(U)-tail.

The S1 motif within *Tb*KREPA4 OB fold was identified using Profile Scan against the protein databases. The motif, which was first identified in the *E. coli* ribosomal protein S1 (Subramanian 1983), is related to the cold shock domain (CSD), and consists of an anti-parallel β -barrel with five β -strands. The S1 motif is present in many proteins with unrelated functions, but all bind RNA (Bycroft et al. 1997). Here we have shown that recombinant KREPA4 interacts with gRNA in vitro. We and others have previously shown (Salavati et al. 2002; Aphasizhev et al. 2003b) that the nuclease treatment of editing complexes shifts the position of the endonuclease, in vitro editing, and RNA ligase activities. These studies have suggested that RNA degradation results in loss of editing complex components. However, our data do not allow us to conclude whether the U specificity of KREPA4 is directed in vivo at the oligo (U) tail or Us added to mRNA or deleted from it. KREPA4 also is a key component of 20S editosomes since its loss results in their disintegration; however KREPA4 does not appear to be a stable component of the deletion and insertion subcomplexes. The S1 motif in polynucleotide phosphorylase stimulates its exonucleolytic degradation of mRNA and may help other molecules involved in RNA degradation. For instance, in *E. coli*, S1 stimulates the activity of T4 phage regB RNA-specific endonuclease (Ruckman et al. 1994). By analogy, KREPA4 may interact with RNA and protein(s) to stimulate or otherwise affect its (their) activity in editing. This is superficially similar to the roles proposed for KREPA1 and KREPA2 in substrate binding and coordination of the order of U addition/ligation and U

deletion/ligation, respectively (Schnauffer et al. 2003). At present the binding partners of KREPA4 in the 20S editosome are unknown but their identification may advance understanding of its role.

The divergence of the *T. cruzi* and *L. major* KREPA4 ortholog (*Tc*KREPA4 and *Lm*KREPA4) sequences from

T. brucei is similar to our previous report that *Tc*KREPA4 and *Lm*KREPA4 proteins have the least homology with the family of OB-fold-containing proteins (Wortheley et al. 2003). *Tc*KREPA4 was identified by homology searches but *Lm*KREPA4 was identified from its syntenic location compared to *T. cruzi* and *T. brucei*. The lack of an apparent S1 motif in *Tc*KREPA4 and *Lm*KREPA4 may reflect development of divergent or novel functions in *T. cruzi* and *L. major* compared to *T. brucei* since paralogs commonly evolve new functions (Kondrashov et al. 2002). The divergence, including that in the S1 motif region, may reflect differences in KREPA4's interactions with RNA and protein among the three species, perhaps to accommodate divergence in other editing proteins and/or RNA substrates or possibly regulatory processes among these species.

Depletion of almost any component of *T. brucei* 20S editosomes (e.g., except for KREL2) results in cell death or a dramatic reduction in the growth rate with associated defects in RNA editing. In some cases, specific editosome components can be depleted without major structural consequences to the 20S editosome and retention of most catalytic activities. However, loss of KREPA4, like KREPB5, has dramatic structural consequences that result in the loss of the editosomes (Fig. 3). Nevertheless, while the levels of the KREPA1, -2, -3, -6, and KREL1 editosome proteins were dramatically decreased in the KREPA4 deficient cells, the MRP1 and REAP-1 proteins of other complexes with possible roles in editing were unaffected. These results suggest a critical role for KREPA4 in the 20S core complex, which might entail editosome assembly or a role associated with the dynamic events that occur in the core complex during editing in *T. brucei*. Its presence in the editosome and the dependence on it for editosome integrity indicates a likely protein interaction domain. However, KREPA4's binding partner(s) and its location in the 20S editosome are as yet unknown. Nevertheless, our results that KREPA4 is required for ~20S editosome integrity suggests an association with both subcomplexes. Thus while KREPA4 does not appear to have a catalytic role in editing, our data suggest it is important for editosome structure and substrate binding.

MATERIALS AND METHODS

Purification of recombinant KREPA4

E. coli BL21 (DE3) pLysS cells (200 mL) containing N-terminal truncated KREPA4 in pRSETc plasmid (Invitrogen) were grown to logarithmic phase in LB and induced with 1 mM IPTG for 3 h. Cells were collected by centrifugation at 6000g for 15 min at 4°C. The cell pellet was then resuspended in 5 mL lysis buffer (50 mM Tris at pH 8.0, 300 mM NaCl, 10 mM imidazole, and 1 mg/mL lysozyme), incubated on ice for 30 min, and sonicated on ice (6 × 10-sec bursts at 200 W with 10-sec cooling period). Lysate was centrifuged at 10,000g for 25 min at 4°C. The supernatant was then transferred to a new tube and an equal volume of 50 mM Tris

(pH 8.0) was added. The cleared lysate was then added to an Invitrogen ProBond Resin Column and rotated 1 h at 4°C. Resin and column were previously prepared by adding 1 mL ProBond resin to the column, washing twice with 10 mL of 20 mM Tris (pH 8.0) and 1 × 5 mL of 20 mM Tris (pH 8.0), 300 mM NaCl, and 10 mM imidazole and then stored at 4°C. The column was drained by gravity flow and washed with 2 × 4 mL wash buffer (20 mM Tris at pH 8.0, 300 mM NaCl, 20 mM imidazole). KREPA4 was eluted by adding 4 × 500 µL elution buffer (20 mM Tris at pH 8.0, 300 mM NaCl, 250 mM imidazole). Glycerol was added to a final concentration of 30%, and samples were stored at -80°C.

KREPA4 gel shift assay

gA6[14] guide RNA, gA6[14] lacking an oligo(U) tail, and A6 pre-edited mRNA (A6 short/TAG.1) transcripts used in this assay were prepared by T7 polymerase (Promega) transcription of PCR-generated templates as previously described (Seiwert et al. 1996), and the 90-nt pBlueScript SK+ (Stratagene) RNA was generated by in vitro transcription of the NotI linearized plasmid. Gel shift assays were performed by incubating 20 ng (800 fmol) of purified KREPA4 with 1 fmol (10K cpm) of internally [α -³²P]UTP-labeled gA6[14] RNA substrate (heated at 95°C for 5 min) in the presence or absence of unlabeled competitor in buffer GS-RBB50 (20 mM Tris-HCl at pH 7.6, 50 mM KCl, 5 mM MgCl₂, 100 µg/mL BSA, 10% glycerol), 100 mM KCl, and 20 units RNasin (Promega) in a 20-µL volume for 30 min at room temperature. In competition experiments, unlabeled gA6[14] guide RNA, gA6[14] RNA lacking an oligo(U) tail, A6short/TAG.1 pre-edited RNA, pBluescript RNA, or poly(N) competitor RNAs were added in 1-, 10-, or 100-fold molar excess to [α -³²P]UTP-labeled gA6[14] RNA followed by the addition of KREPA4. Samples were then loaded onto 9% native polyacrylamide minigels (Bio-Rad) in 0.5× TBE and run at 100 V for 1 h at 4°C. Gels were dried and visualized on Storm PhosphorImager screens (Molecular Dynamics). The intensity of the bands was quantified using ImageQuant software.

Plasmid constructs, transfections, and induction of RNAi

Three KREPA4 dsRNA-containing vectors were constructed that contained either the full-length KREPA4 ORF, the 5' 315 bp including 60 bp of the 5' UTR, or the 3' 327 bp of KREPA4. The inserts were PCR amplified from *T. brucei* PF 29.13 genomic DNA. The 657-bp full-length ORF was amplified using 5'-CCG CTCGAGCGGATGCGGGTGCGTTCACCTCCT-3' as the forward primer and 5'-AACTGCAGAACCAATGCATTGGTTAACTC CAACCTCCTGC-3' as the reverse primer, the 315-bp 5' fragment was amplified using 5'-CCGCTCGAGCGGTCTCTGAAGGAAA GGTGTGT-3' as the forward primer and 5'-CCCAAGCTTGGGC GTGAGGCAAATTGGATTGCGCC-3' as the reverse primer, and the 327-bp 3' fragment was amplified with 5'-CCGCTCGAGCGG TTGGTGCATCGCCAACCTGTGAC-3' as the forward primer and 5'-CCCAAGCTTGGGGTGCACGTAGCGGGGAGACCA-3' as the reverse primer. The resulting PCR products were digested with XhoI or PstI and HindIII and ligated into the pZJM vector (Wang et al. 2000) that had also been digested with compatible restriction enzymes to create the final constructs.

The 29.13 strain of PF *T. brucei* that has integrated genes for T7 RNA polymerase and the tetracycline (tet) repressor was used for

all of the transfections (Wirtz et al. 1999). Plasmid DNA (15 µg) was linearized with NotI, and cells were transfected and cloned 1 d after the transfection by limiting dilution. The cells were grown in SDM-79 medium at 27°C and the transfectants were selected in 25 µg/mL hygromycin, 15 µg/mL neomycin, and 2.5 µg/mL phleomycin. Double-stranded RNA was induced with 1 µg/mL tet and the uninduced and induced cells were counted daily to obtain growth curves. The cells were maintained between 1.0×10^6 and 2.0×10^7 cells/mL throughout the course of RNAi induction. The cumulative cell number was determined by multiplying the number of cells by the dilution.

RNA analysis

Trizol (Invitrogen) reagent was used to isolate total RNA from the parasites. Northern blot analysis was performed on 10 µg RNA from cells that were grown for 2 or 4 d in the absence and presence of 1 µg/mL of tet. Preparation of the RNA, transfer of the RNA to the membrane (Hybond-N+, Amersham Pharmacia Biotech), and hybridization and detection were performed according to the manufacturer's protocol (ULTRAhyb, Ambion). The oligonucleotide complementary to the 5' UTR region of KREPA4 used for Northern analysis was TAATACGACTCACTATAGGGG GACTGAGCACTGCATCAA.

Real-time PCR

Real-time PCR was carried out essentially as described (Carnes et al. 2005). Total RNA was isolated from cells using Trizol LS Reagent (Invitrogen) and 10 µg were DNase I treated using the DNafree kit (Ambion). The integrity of the RNA was confirmed using an RNA nanochip on a BioAnalyzer (Agilent Technologies). The cDNA templates for real-time PCR were reverse transcribed from 4.5 µg of RNA using random hexamers and Taqman Reverse Transcription Reagents (Applied Biosystems) in a 30 µL reaction. Each experiment had a reaction without reverse transcriptase as a control. The 30 µL reaction was then diluted sevenfold in water. For each PCR reaction, 12.5 µL of SYBR Green Master Mix (Applied Biosystems), 5 µL of 1.5 µM forward oligo, 5 µL of 1.5 µM reverse oligo, and 2.5 µL cDNA template (or -RT control) were combined in a well of a 96-well plate (Applied Biosystems). ABI Prism 7000 thermocycler conditions for all reactions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Template was further diluted 1:50 for the 18S rRNA internal control so C_t values were similar to less abundant edited and pre-edited RNAs. Thermal dissociation confirmed the PCR generated a single amplicon. Primers for real-time PCR were designed using ABI Primer Express v2.0 software. The sequences of most of the primers were described previously (Carnes et al. 2005) except KREPA6 forward: TTGTT GCGTTCGTTTCATGAT and KREPA6 reverse: TGCGTGACGG CATCTTCATA. Relative changes in target amplicons were determined using the Pfaffl method, with PCR efficiencies calculated by linear regression using LinRegPCR.

Assays of enzymatic activities

Precleaved deletion editing specifying the removal of four Us and insertion editing specifying the addition of two Us were assayed *in vitro* with 5'-labeled U5 5'CL, U5 3'CL with gA6[14]PC-del and

5'-labeled 5'CL18, 3'CL13pp with gPCA6-2A RNAs, respectively (Igo et al. 2000, 2002). The reaction products were resolved on polyacrylamide-urea gels and visualized on Storm PhosphorImager screens (Molecular Dynamics). RNA ligases were adenylated as previously described (Sabatini and Hajduk 1995) in 25 mM HEPES, pH 7.9, 10 mM Mg(OAc)₂, 50 mM KCl, 0.5 mM DTT, and 10% dimethylsulfoxide with 2.5 µCi [α -³²P]ATP. The proteins were resolved by 10% SDS-PAGE gels and the radiolabeled proteins were detected by PhosphorImaging.

Analysis of the editing complex

TAP-tagged KREPA4 complexes were purified from 2×10^9 cells as previously described (Rigaut et al. 1999; Schnauffer et al. 2003). For glycerol gradient analysis, cells were lysed in 1 mL of Lysis buffer (20 mM HEPES at pH 7.9, 10 mM Mg(OAc)₂, 50 mM KCl, 1 mM EDTA, 1% Triton-X) containing protease inhibitors (1 µg/mL Pepstatin, 2 µg/mL Leupeptin, 1 mM Pefabloc). Centrifugation was used to clear the lysate, and an equal amount of protein was then loaded onto an 11 mL 10%–30% glycerol gradient. The gradients were centrifuged at 38,000 rpm for 5 or 9 h at 4°C (SW40 rotor; Beckman), 500-µL fractions were collected from the top, flash frozen in liquid nitrogen, and stored at –80°C. Every other fraction from the gradient (33 µL) was resolved on 10% SDS-PAGE gels. The proteins were transferred onto PVDF membranes and reacted with MAbs specific for KREPA1–3, KREL1 (Panigrahi et al. 2001a), REAP1 (Madison-Antenucci et al. 1998), and MRP1 (Allen et al. 1998), or KREPA6 (Schnauffer et al. 2003) and PGK (Parker et al. 1995) polyclonal antibodies. The membranes were developed with the ECL kit (Amersham) as per the manufacturer's instructions.

KREPA4 modeling

A structural model of KREPA4 was built with the MODELLER program using software programs from Accelrys Inc., DS Modeling 1.1 (Sali and Blundell 1993). The model was generated based on the multiple alignments shown in Figure 5, using the S1 motifs from *E. coli* PNPase (PDB code 1sro) and *E. coli* RNase E (PDB code 1smx) as templates. The quality of the modeled structure was checked with the Profiles_3D program (Bowie et al. 1991) in DS Modeling 1.1.

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