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RBP16 stimulates trypanosome RNA editing in vitro at an early step in the editing reaction

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ABSTRACT

RBP16 is an abundant RNA binding protein from *Trypanosoma brucei* mitochondria that affects both RNA editing and stability. We report here experiments aimed at elucidating the mechanism of RBP16 function in RNA editing. In in vitro RNA editing assays, recombinant RBP16 is able to significantly stimulate insertion editing of both CYb and A6 pre-mRNAs. Enhancement of in vitro editing activity occurs at, or prior to, the step of pre-mRNA cleavage, as evidenced by increased accumulation of pre-mRNA 3′ cleavage products in the presence of RBP16. Mutated RBP16 that is severely compromised in cold shock domain (CSD)-mediated RNA binding was able to enhance editing to levels comparable to the wild-type protein in some assays at the highest RBP16 levels tested. However, at low RBP16 concentrations or in assays with native, oligo(U)-tail-bearing gRNAs, editing stimulation by mutant RBP16 was somewhat compromised. Together, these results indicate that both the N-terminal CSD and C-terminal RGG RNA binding domains of RBP16 are required for maximal editing stimulation. Finally, the relaxed specificity of RBP16 for stimulation of both CYb and A6 editing in vitro implicates additional specificity factors that account for the strict CYb specificity of RBP16 action in editing in vivo. Our results constitute the first report of any putative RNA editing accessory factor eliciting an effect on editing in vitro. Overall, these results support a novel accessory role for RBP16 in U insertion editing.

Keywords: RNA editing; trypanosome; mitochondria; Y-box protein; guide RNA

INTRODUCTION

RNA editing in the mitochondria of trypanosomatids entails the guided insertion and deletion of uridine residues into mitochondrial pre-mRNAs (for reviews, see Simpson et al. 2004; Stuart et al. 2005). The majority of transcripts encoded on the maxicircle component of trypanosome mitochondrial DNA require this unusual form of post-transcriptional gene regulation to create functional open reading frames. The editing process is catalyzed by a series of enzymes contained within a large multiprotein complex termed the editosome (see below). Guide RNAs (gRNAs), which are encoded on mitochondrial DNA molecules termed minicircles, are small *trans*-acting molecules that confer the genetic information required for editing to the mRNA through base-pairing interactions.

A cycle of RNA editing involves three major enzyme-catalyzed reactions. After formation of the gRNA/mRNA anchor duplex, the mRNA is cleaved by a gRNA-directed endonuclease 5′ of the anchor region. In the case of a U insertion event, U residues are added to the 3′ end of the mRNA 5′ cleavage fragment by terminal uridylyl transferase (TUTase). In the case of a U deletion event, U residues are removed from the 3′ end of the 5′ cleavage fragment by a U-specific 3′–5′ exonuclease. After U insertion or deletion has taken place, the mRNA cleavage fragments are rejoined by RNA ligase. Editing of the pre-mRNA extends the complementarity of the gRNA–mRNA duplex, and gRNA–mRNA mismatches continue to direct pre-mRNA cleavage and subsequent rounds of editing until complete gRNA–mRNA complementarity is achieved. Occasionally, gRNA–mRNA "chimeras" formed by ligation of gRNA to the 5′ end of the mRNA 3′ cleavage fragment are detected in vivo (Blum et al. 1992). Chimeras are nonproductive byproducts of the

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editing reaction and are also commonly detected in in vitro RNA editing reactions (Seiwert et al. 1996).

Multiprotein editosome complexes capable of supporting a full cycle of U insertion or deletion in vitro have been enriched from the mitochondria of trypanosomatids by several laboratories (Rusché et al. 1997; Madison-Antenucci et al. 1998; Panigrahi et al. 2001; Aphasizhev et al. 2003a). Editosomes sediment at ~20S on glycerol gradients and contain the four enzymatic activities required for editing. The polypeptides that catalyze several of these enzymatic reactions have been identified (Carnes et al. 2005; Kang et al. 2005; Stuart et al. 2005; Trotter et al. 2005). Additional proteins containing ribonuclease and/or RNA binding motifs are also present within the complex, although their roles are not fully understood. Interestingly, editosome proteins exist in sets or pairs that are related by their sequence characteristics (Simpson et al. 2004; Stuart et al. 2005). This reflects, in part, the separation of the editosome into functionally and structurally distinct insertion and deletion subcomplexes (Schnaufer et al. 2003). The efficiency of the editing process is dependent on the regulated assembly of pre-mRNA, gRNA, and protein components associated with the catalysis and/or regulation of the process. Thus, along with the catalytic polypeptides of the editing machinery, additional editosome components presumably promote RNA binding and positioning relative to the enzyme active sites. Accessory factors involved in regulating the specificity, accuracy, and/or efficiency of the process may also transiently interact with the editosome. Hence, the editosome is likely to be a dynamic complex containing proteins that are loosely or transiently associated with a stable core catalytic complex (Stuart et al. 2005).

RBP16 is an RNA binding protein from *Trypanosoma brucei* mitochondria that was initially identified in a search for protein factors that specifically bind gRNAs (Hayman and Read 1999). In vitro, this 16-kDa protein binds different gRNAs primarily via the 3′ oligo(U) tail (Hayman and Read 1999; Pelletier et al. 2000). RBP16 was later shown to be associated with gRNAs in vivo through both immunoprecipitation and *in organello* cross-linking studies (Hayman and Read 1999; Militello et al. 2000). In addition to binding gRNAs, RBP16 can also be found associated with 9S and 12S rRNAs and a subset of mRNAs in *T. brucei* mitochondria (Hayman and Read 1999; M. Pelletier and L. Read, unpubl. results). The RBP16 protein contains a conserved nucleic acid binding domain known as the cold shock domain (CSD) at its N terminus, placing RBP16 as a member of the eukaryotic Y-box protein family (Graumann and Marahiel 1996; Hayman and Read 1999). Y-box proteins comprise a family of DNA and RNA binding proteins involved in pleiotropic biological functions, including regulation of gene expression at the transcriptional (Mertens et al. 1997; Norman et al. 2001; Safak et al. 2002), post-transcriptional (Grosset et al. 2000; Evdokimova et al. 2001; Stickeler et al. 2001), and translational levels (Sommerville and Ladomery 1996; Evdokimova et al. 1998; Pisarev et al. 2002). In addition, some CSD-containing proteins have been reported to facilitate nucleic acid annealing (Skabkin et al. 2001) or to destabilize RNA secondary structure by acting as RNA chaperones (Jiang et al. 1999; Bae et al. 2000). At its C terminus, RBP16 contains a domain rich in arginine and glycine residues, resembling an RGG RNA binding motif (Burd and Dreyfuss 1994; Hayman and Read 1999). Taken together, the inclusion of RBP16 in the multifunctional Y-box protein family and the diverse RNA binding properties of RBP16 suggest multiple functions for RBP16 in the regulation of mitochondrial gene expression in *T. brucei*.

RNA interference (RNAi) studies from our laboratory previously provided strong evidence that RBP16 plays a role in RNA editing in vivo (Pelletier and Read 2003). Disruption of RBP16 expression in procyclic form *T. brucei* resulted in the specific, dramatic reduction of edited apocytochrome b (CYb) mRNA levels by 98% with little effect on other edited mRNAs. A concomitant increase in unedited CYb mRNA levels strongly suggests that RBP16 plays a critical role in the editing of CYb mRNA. In addition to its role in editing, RBP16 also modulates RNA stability, as evidenced by the significant decrease in abundance of two never edited mRNAs paralleling RBP16 depletion. The ability of RBP16 to modulate specific RNA editing events is presumably mediated through its interaction with gRNAs and/or mRNAs. The mRNA binding capacity of the protein is also likely to account for its role in regulation of mRNA stability, while rRNA binding suggests additional regulatory functions. The demonstration that RBP16 is essential for CYb RNA editing defined this protein as the first reported editing accessory factor in trypanosomes. Subsequently, disruption of the MRP1/2 complex was reported to have a remarkably similar effect on both RNA editing and stability in *T. brucei* (Vondrusková et al. 2005). Current evidence indicates that RBP16 does not stably interact with the MRP1/2 complex or its constituent proteins (Schnaufer et al. 2003; Vondrusková et al. 2005; C. Goulah and L. Read, unpubl. results). Indeed, the molecular mechanism by which RBP16 facilitates the RNA editing process is completely unknown.

To investigate the mechanistic role of RBP16 in CYb editing, we utilized an in vitro RNA editing assay based on the first editing site (ES1) of CYb pre-mRNA (Igo et al. 2002). Recombinant RBP16 was titrated into in vitro insertion assays under various conditions of preincubation with editing reaction components. We show that RBP16 causes a dramatic stimulation of CYb insertion editing in vitro, enhancing both edited product and 3′ cleavage product formation. We also observed RBP16-mediated enhancement of in vitro insertion activity at ES2 of the ATPase subunit 6 (A6) pre-mRNA, although stimulation of A6 editing was reduced compared to the stimulation of CYb editing. Analysis of editing intermediates demonstrates

that RBP16-mediated enhancement of CYb and A6 RNA editing apparently occurs by a similar mechanism. Because RBP16 is able to affect the editing in vitro of an mRNA whose editing is unaffected by RBP16 depletion in vivo, this potentially implicates additional sequence-specific regulatory factors in RBP16-mediated enhancement of CYb editing in vivo. Studies with a mutant version of RBP16 compromised in RNA binding ability suggest that editing stimulation does not strictly require binding of the RBP16 CSD to the gRNA oligo(U) tail, although several lines of evidence point to a contribution of this interaction. The data presented here constitute the first report of any putative RNA editing accessory factor eliciting an effect on editing in vitro. Overall, these results are consistent with our previous analysis of RNA editing in RBP16-depleted cells (Pelletier and Read 2003) and support an accessory role for RBP16 in U insertion editing.

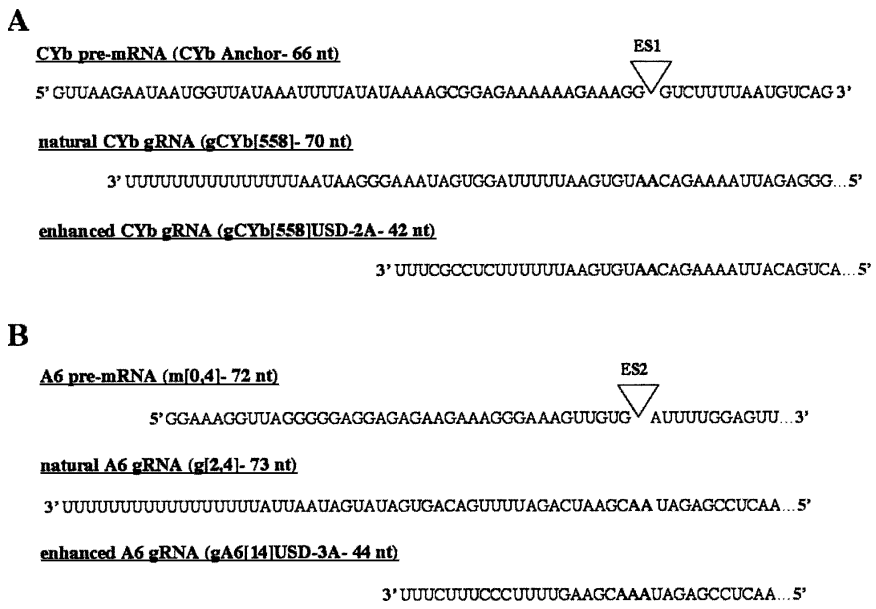

FIGURE 1. RNAs utilized in in vitro editing assays. Common names of RNAs are followed by published names (Riley et al. 1994; Cruz-Reyes and Sollner-Webb 1996; Cruz-Reyes et al. 1998, 1998; Igo et al. 2002) and lengths of specific constructs in parenthesis. (*A*) CYb RNAs. The full sequence of the CYb pre-mRNA construct is shown in a 5′–3′ direction. Partial sequences of CYb gRNA constructs are matched up with the CYb pre-mRNA sequence and are presented in a 3′–5′ direction. The site of the CYb pre-mRNA that is edited by insertion of U residues in the in vitro assay (ES1) is indicated by an arrowhead. The corresponding A residues in the CYb gRNAs that specify the number of uridines inserted (two for both natural and enhanced CYb gRNAs) are indicated in bold. (*B*) A6 RNAs. A partial sequence of the A6 pre-mRNA construct is shown in a 5′–3′ direction. Partial sequences of A6 gRNA constructs are matched up with the A6 pre-mRNA sequence and are presented in a 3′–5′ direction. The site of the A6 pre-mRNA that is edited by insertion of U residues in the in vitro assay (ES2) is indicated by an arrowhead. The corresponding A residues in the A6 gRNAs that specify the number of uridines inserted (two and three for the natural and enhanced A6 gRNAs, respectively) are indicated in bold.

RESULTS

Effect of RBP16 on insertion editing of CYb pre-mRNA in vitro

To begin to address the mechanism by which RBP16 facilitates RNA editing, we asked whether addition of RBP16 to in vitro editing assays had a stimulatory effect on edited RNA production. Because RBP16 depletion dramatically and specifically inhibits editing of CYb RNA in vivo (Pelletier and Read 2003), we began by utilizing an in vitro RNA editing assay based on the first editing site (ES1) of CYb pre-mRNA (Fig. 1A; Igo et al. 2002). The natural CYb transcript is edited only in its 5′ region exclusively by insertion events and is edited solely in PF *T. brucei*. The natural CYb gRNA (gCYb[558]) (Riley et al. 1994), which specifies the insertion of two uridines into ES1, does not support in vitro editing activity (Igo et al. 2002). To determine whether addition of RBP16 would increase the efficiency of the reaction with native CYb gRNA, we titrated RBP16 into insertion editing assays with CYb pre-mRNA (CYb Anchor) and the natural CYb gRNA (gCYb[558]) (data not shown). Titration of RBP16 into editing assays resulted in increased production of a band larger than the expected size. Instead of obtaining a +2U product, we obtained a band that was 4 nt larger than the input RNA. Cloning and sequencing of this product revealed a gRNA–mRNA chimera in which the mRNA 3′ cleavage fragment was ligated to a gRNA lacking a U tail. In addition, chimeras were obtained with rearranged gRNA regions. Hence, we were unable to determine parameters of RBP16 function in vitro using the native CYb RNAs.

Since the natural CYb gRNA is unable to support in vitro insertion even in the presence of RBP16, the enhanced CYb gRNA gCYb[558]USD-2A, which also specifies insertion of two uridines into ES1, was tested as an alternative (Fig. 1A; Igo et al. 2002). The enhanced CYb gRNA, which has been altered to support in vitro editing activity, is modified so that its 3′ region precisely base-pairs with the CYb pre-mRNA region just upstream of the editing site, resulting in a more stable mRNA–gRNA association. In addition, the information region of the enhanced gRNA has been shortened and the complementarity of its 5′ anchor region to the pre-mRNA has been extended. Because the modifications of the 3′ end of the enhanced CYb gRNA interrupt the natural oligo(U) tail, which is the primary determinant for RBP16-gRNA binding (Hayman and Read 1999; Pelletier et al. 2000), we first wanted to determine the affinity of RBP16 for the enhanced gRNA relative to the other RNA

substrates utilized in the editing assay. Titration of RBP16 (0.5–10 μM) into electrophoretic mobility shift assays (EMSAs) with the three CYb RNAs demonstrated that RBP16 was able to bind to the enhanced CYb gRNA, albeit with lower affinity than to the natural CYb gRNA or CYb pre-mRNA (Fig. 2). While lacking a typical oligo(U) tail, the enhanced CYb gRNA contains a U-rich region at its 3′ end that may be sufficient for RBP16 binding (Fig. 1A). The ability of RBP16 to bind the enhanced CYb gRNA is not unexpected given our finding in a previous study that RBP16 can achieve efficient binding of a stretch of four U's when sufficient sequence 5′ and 3′ of the binding site are present (Pelletier et al. 2000).

To determine whether RBP16 enhances the editing of CYb RNA in vitro, we titrated RBP16 (0.5–10 μM) into CYb insertion assays utilizing the enhanced CYb gRNA (Fig. 3). The level of edited RNA (+2U) was quantitated and expressed as the fraction of input RNA converted to product normalized to the amount obtained in the absence of additional protein (Fig. 3B). No chimera formation was detected with this pre-mRNA–gRNA pair, presumably due to a more stable mRNA–gRNA association compared to the natural CYb gRNA. As shown in Figure 3, addition of RBP16 to CYb insertion assays elicited a reproducible three- to fivefold enhancement of edited RNA formation in a somewhat concentration-dependent manner. We also assessed production of pre-mRNA 3′ cleavage products. These RNAs are generated during the initial endonucleolytic step in the editing reaction and typically accumulate during in vitro editing reactions. Formation of the pre-mRNA 3′ cleavage product was also stimulated approximately threefold by addition of RBP16 (Fig. 3). Acetylated BSA, in an approximately equal mass amount of protein relative to the highest concentration of RBP16 tested (10 μM), failed to stimulate formation of either fully edited RNA or 3′ cleavage product under any conditions. Polyethylene glycol (PEG), a molecular crowding agent, was also titrated into in vitro insertion reactions as an additional control. PEG is often used to control for nonspecific effects resulting from volume exclusion due to addition of exogenous protein. It, too, had no significant effect on either edited RNA or cleavage product formation. Overall, the results from this experiment demonstrate that RBP16 stimulates in vitro editing of CYb pre-mRNA at least partially through an effect at, or prior to, pre-mRNA cleavage.

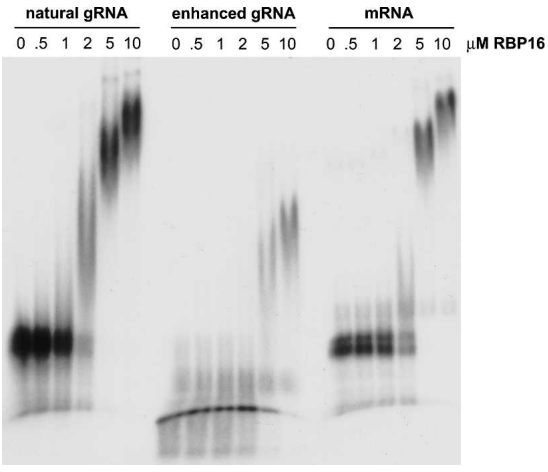

FIGURE 2. EMSA analysis of the RNA binding affinity of RBP16 for RNA substrates utilized in CYb insertion assay. Increasing concentrations of recombinant RBP16 were incubated with 5 fmol of internally radiolabeled RNA. Following electrophoresis on an 8% nondenaturing acrylamide gel, RNA–protein complexes were detected by autoradiography.

To more fully investigate the mechanism by which RBP16 stimulates in vitro insertion activity, we preincubated RBP16 with various components of the editing reaction (gRNA or mRNA) prior to addition of the remainder of the components. In general, preincubation of RBP16 with either gRNA or mRNA did not significantly alter the degree of editing stimulation compared to reactions with no preincubation (cf. Figs. 3 and 4). These experiments suggest that RBP16-RNA binding prior to the initiation of the editing reaction does not additionally enhance the degree of RNA editing in vitro under the conditions tested. However, we cannot rule out from these experiments that preincubation of RBP16 with RNA components of the assay may have increased the initial rate of the reaction.

Mutation of RBP16 cold shock domain

We previously showed that RBP16 contains two distinct RNA binding domains, the CSD and the RGG domain (Miller and Read 2003). The CSD interacts with the oligo(U) tail, thereby contributing the majority of the affinity and specificity of RBP16–gRNA binding. The RGG domain further stabilizes the interaction through nonspecific contacts with the encoded portion of the gRNA (Pelletier et al. 2000; Miller and Read 2003). We next wanted to directly examine the role of CSD-oligo(U) tail interactions in the overall effect of RBP16 on in vitro insertion editing. To this end, we mutated phenylalanines 14 and 16 of the RBP16 RNP1 RNA binding motif (which is contained within the CSD) to alanines to create RBP16(F14,16A) (Fig. 5A). Based on experiments with other CSD-containing proteins, these mutations were predicted to drastically reduce the RNA binding capacity of RBP16 (Bouvet et al. 1995; Schroder et al. 1995). We confirmed the compromised ability of RBP16(F14,16A) to bind the RNA substrates utilized in the in vitro insertion assay by EMSA (Fig. 5B; data not shown). In contrast to RBP16, RBP16(F14,16A) was unable to form a stable protein–RNA complex with the enhanced CYb gRNA at the range of protein concentration tested (Fig. 5B, 0.5–10 μM). However, RBP16(F14,16A) (10 μM) retained a fraction of its RNA binding activity under UV cross-linking conditions (Fig. 5C). We attribute

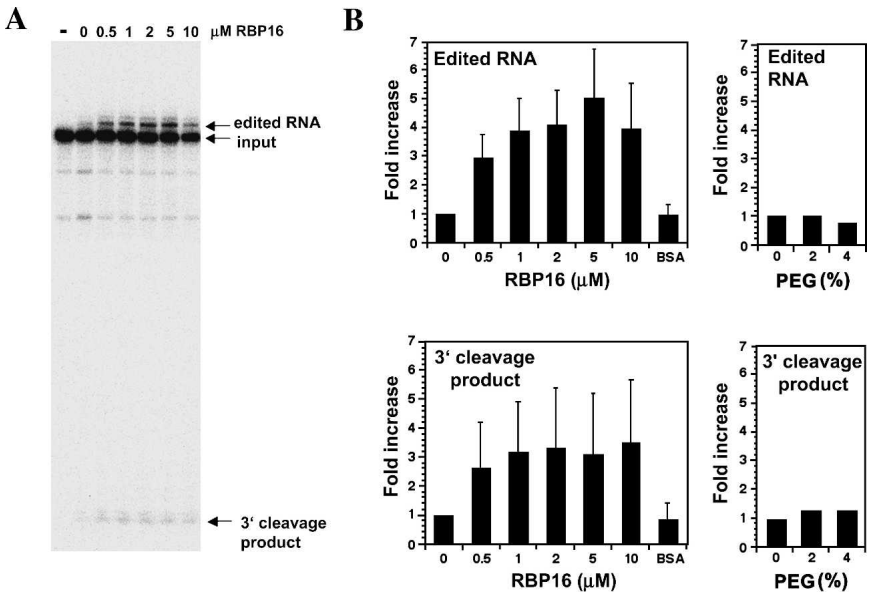

FIGURE 3. Effect of RBP16 on in vitro insertion editing of CYb with enhanced gRNA. RBP16 (0.5–10 μM) was titrated into CYb in vitro insertion reactions as described in Materials and Methods. (*A*) Reactions were visualized by gel electrophoresis followed by phosphorimager analysis. Positions of input RNA, edited (+2U) RNA, and 3′ cleavage products are indicated. Lane denoted (−) is equivalent to 0 lane, except that gRNA was omitted as a negative control. (*B*) Edited RNA and pre-mRNA 3′ cleavage products were quantitated by phosphorimager. Data are expressed as the fraction of input RNA converted to each product normalized to the amount obtained in the absence of additional protein (0) (i.e., fold increase over no RBP16 levels). Results from three independent experiments are displayed as mean and standard deviation. BSA (150 ng/μL) was included as a negative control and represents an approximately equal mass amount of protein relative to the highest concentration of RBP16 tested (10 μM). PEG (2% or 4%) was titrated into in vitro insertion reactions to control for nonspecific effects due to molecular crowding.

this residual level of RNA binding activity to the RGG domain of RBP16. We previously showed that the RGG domain alone is unable to stably interact with RNA under EMSA conditions but is able bind to RNA in UV cross-linking assays (Miller and Read 2003).

To assess the role of CSD–RNA interactions in editing stimulation, we titrated RBP16(F14,16A) into CYb insertion assays under the same conditions tested with RBP16 (Fig. 6). Surprisingly, at the higher protein concentrations, RBP16 (F14,16A) was able to enhance CYb insertion editing as well as wild-type RBP16 (cf. Figs. 3 and 6, and 2–10 μM). However, at lower protein concentrations, the ability of RBP16(F14,16A) to stimulate edited RNA formation appeared decreased compared to wild-type RBP16 (cf. Figs. 3 and 6, 0.5 and 1 μM). Statistical analysis confirmed that wild-type RBP16 was significantly more effective than mutant RBP16 in its ability to stimulate CYb editing at both 0.5 and 1 μM concentrations ($p < 0.05$). In addition, stimulation of edited product formation by RBP16(F14,16A) was non-saturable at the range of protein concentrations tested, whereas enhancement by wild-type RBP16 was saturated at 2 μM (cf. Figs. 3 and 6, Edited RNA). This pattern may reflect the contribution of nonspecific, low affinity protein–RNA contacts in the absence of CSD–RNA binding, thereby preventing RBP16 from saturating the RNA substrate. 3′ cleavage product formation was also stimulated by RBP16(F14,16A), and this effect was also not saturable under these assay conditions, in contrast to wild-type RBP16 (cf. Figs. 3 and 6, 3′ cleavage product). Hence, CSD–oligo(U) tail interactions are not required for RBP16 editing stimulation in vitro, but the CSD does contribute to some parameter(s) of the enhancement. Together, these results suggest both the CSD and RGG domains contribute to the ability of RBP16 to stimulate CYb RNA editing.

Effect of RBP16 on insertion editing of A6 pre-mRNA in vitro

The results obtained from the in vitro CYb insertion assay are consistent with in vivo data from RBP16 knockdown cells (Pelletier and Read 2003). Both sets of results point to a role for RBP16 in facilitating the editing of CYb pre-mRNA. We next wanted to determine whether the in vivo specificity of RBP16 CYb editing was reflected in vitro. With this aim in mind, we made use of an in vitro U-insertional RNA editing assay based on the second editing site (ES2) of A6 pre-mRNA (see Fig. 1B; Cruz-Reyes and Sollner-Webb 1996; Cruz-Reyes et al. 2001; Igo et al. 2002), since A6 editing was unaffected in RBP16 knockdown cells. We titrated RBP16 into A6 insertion editing assays utilizing the A6 (m[0,4]) pre-mRNA and either the natural gRNA g[2,4] (Cruz-Reyes et al. 2001) or the enhanced gRNA gA6[14]USD-3A (Igo et al. 2002). The natural A6 gRNA contains a 17-nt 3′ oligo(U) tail. In the enhanced A6 gRNA, the oligo(U) tail as well as a portion of the information region have been replaced with sequence complementary to part of the upstream purine-rich region of the A6 pre-mRNA. Although both A6 gRNAs support in vitro editing activity, the natural A6 gRNA is less efficient due to weaker interaction with the A6 pre-mRNA, allowing diversion of RNA substrates into the defunct chimera pathway. The A6 assay presents an opportunity to compare the natural gRNA containing an oligo(U) tail to the enhanced gRNA lacking an extended oligo(U) tail, to better elucidate the role of gRNA binding by the CSD of RBP16 in RNA editing enhancement. The relative affinity of RBP16 for the RNA substrates utilized in the A6 insertion assay was first assayed by EMSA. Similar to what was observed with the CYb RNAs, RBP16 was able to

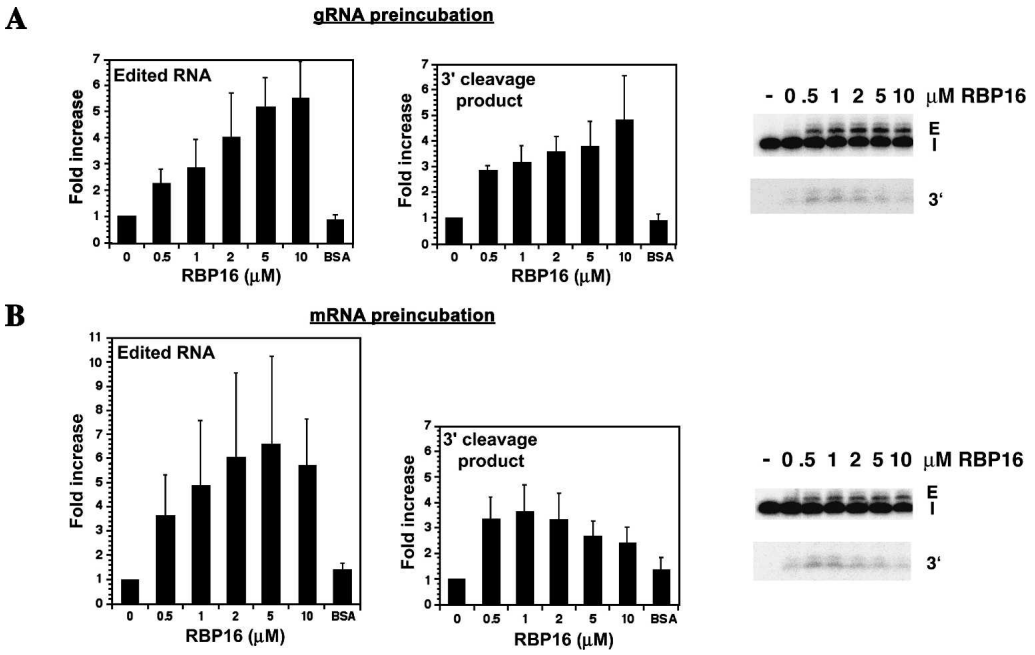

FIGURE 4. Effect of RBP16 on in vitro insertion editing of CYb with enhanced gRNA after preincubation with various RNAs. (*A*) Preincubation with gRNA. RBP16 (0.5–10 μM) was combined with enhanced CYb gRNA and incubated for 5 min at room temperature prior to initiation of the editing reaction. (*B*) Preincubation with mRNA. RBP16 (0.5–10 μM) was combined with CYb pre-mRNA and incubated for 5 min at room temperature prior to initiation of the editing reaction. Quantitation of edited products and 3′ cleavage products is expressed in graphical form on the *left* as described in Figure 3, and a representative experiment is shown on the *right*. (E) edited RNA; (I) input RNA; (3′) 3′ cleavage product. Lane denoted (−) is equivalent to 0 lane, except that gRNA was omitted as a negative control.

bind the enhanced A6 gRNA, although with a lower affinity than the natural A6 gRNA or A6 pre-mRNA (data not shown). Like the enhanced CYb gRNA, the enhanced A6 gRNA also contains a U-rich stretch in its 3′ region that is presumably sufficient for RBP16 binding (Fig. 1B). As expected, no interaction of RBP16(F14,16A) with any of the A6 RNAs was detected by EMSA (data not shown).

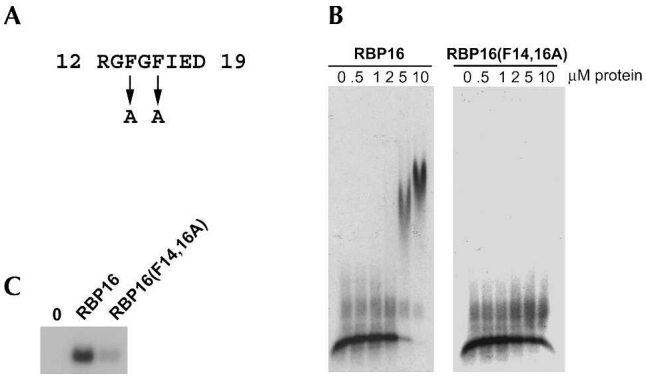

FIGURE 5. RNA binding affinity of RBP16 and RBP16(F14,16A) for the enhanced CYb gRNA. (*A*) The RNP1 RNA binding motif within the CSD of RBP16. Mutations of amino acids 14 and 16 to create RBP16(F14,16A) are indicated. (*B*) EMSA analysis. Increasing concentrations of recombinant RBP16 or RBP16(F14,16A) were incubated with 5 fmol of internally radiolabeled gRNA. Following electrophoresis on an 8% nondenaturing acrylamide gel, RNA–protein complexes were detected by autoradiography. (*C*) UV cross-linking analysis. RBP16 or RBP16(F14,16A) (10 μM) was incubated with 5 fmol internally radiolabeled gRNA as in EMSAs. RNA–protein complexes were UV cross-linked and analyzed by SDS-PAGE and autoradiography as described in Materials and Methods. (Lane *0*) no protein.

Titration of RBP16 into A6 insertion editing reactions with the natural gRNA resulted in stimulation of edited product formation up to 2.5-fold in a concentration-dependent manner (Fig. 7A). This experiment demonstrates that RBP16 is able to stimulate editing in vitro of an mRNA whose editing is unaffected by RBP16 depletion in vivo, suggesting that RBP16 may be able to perform a role in vitro that is normally executed by another factor in vivo. Alternatively, additional specificity factors may interact with RBP16 in vivo, either promoting interaction with CYb RNAs or precluding interaction with A6 RNAs. However, the ability of RBP16 to stimulate CYb and A6 editing is not equivalent. It is clear that the ability of RBP16 to stimulate editing of A6 RNA is significantly reduced compared to its ability to stimulate CYb editing (cf. Figs. 3 and 7, Edited RNA). CYb and A6 editing assays were performed under slightly different conditions that were determined to be optimal for each substrate (data not shown). Nevertheless, the addition of nonspecific RNA in the CYb assays might have been expected to decrease RBP16 action through titration of the protein by nonspecific RNA binding. However, the opposite is observed, suggesting that in vitro CYb editing is intrinsically somewhat more sensitive to

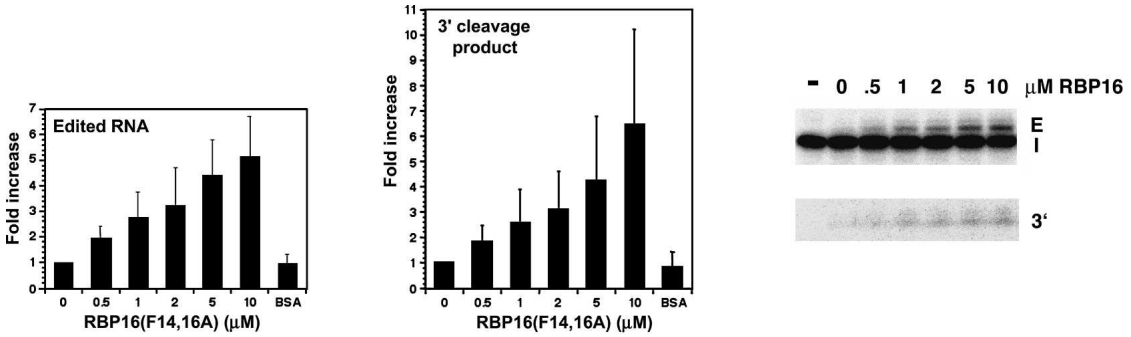

FIGURE 6. Effect of RBP16(F14,16A) on in vitro insertion editing of CYb with enhanced gRNA. RBP16(F14,16A) (0.5–10 μM) was titrated into in vitro insertion reactions. Quantitation of edited RNA and 3′ cleavage products is expressed in graphical form as described in Figure 3. A representative experiment, labeled as in Figure 4, is shown on the *right*.

RBP16 stimulation than is A6 in vitro editing. We also assessed the formation of 3′ cleavage products and chimeras using the A6 assay with the natural gRNA. 3′ cleavage product formation was enhanced two- to fourfold at most protein concentrations, and up to approximately eightfold at the highest RBP16 concentration tested (Fig. 7A, 3′ cleavage product). Chimera formation was stimulated up to six- to sevenfold in a concentration-dependent manner (Fig. 7A, Chimera). In general, these results demonstrate that RBP16 has a broad capacity to stimulate RNA editing in vitro.

We next tested the ability of RBP16(F14,16A) to enhance A6 insertion assays in the presence of the natural gRNA. RBP16(F14,16A) stimulated editing slightly under these conditions, but to a lesser degree than wild-type RBP16 (Fig. 7, cf. A and B, Edited RNA). Maximal stimulation of edited RNA production by RBP16(F14,16A) in the presence of the natural gRNA was just twofold at the highest concentration tested, and only up to 1.5-fold at lower concentrations. Stimulation by mutant RBP16 was shown to differ significantly ($p < 0.05$) from stimulation by wild-type RBP16 under the same conditions at three of the five concentrations tested (0.5, 2, and 10 μM). 3′ cleavage product and chimera formation also appeared somewhat decreased with RBP16(F14,16A) compared to wild-type RBP16. These results indicate that critical mutations within the CSD render RBP16 somewhat inefficient in stimulating editing in the presence of the natural oligo(U) tail-containing A6 gRNA.

To further assess the role of the oligo(U) tail in RBP16 editing stimulation, we assayed A6 editing using the enhanced gRNA, which does not contain a contiguous oligo(U) tail

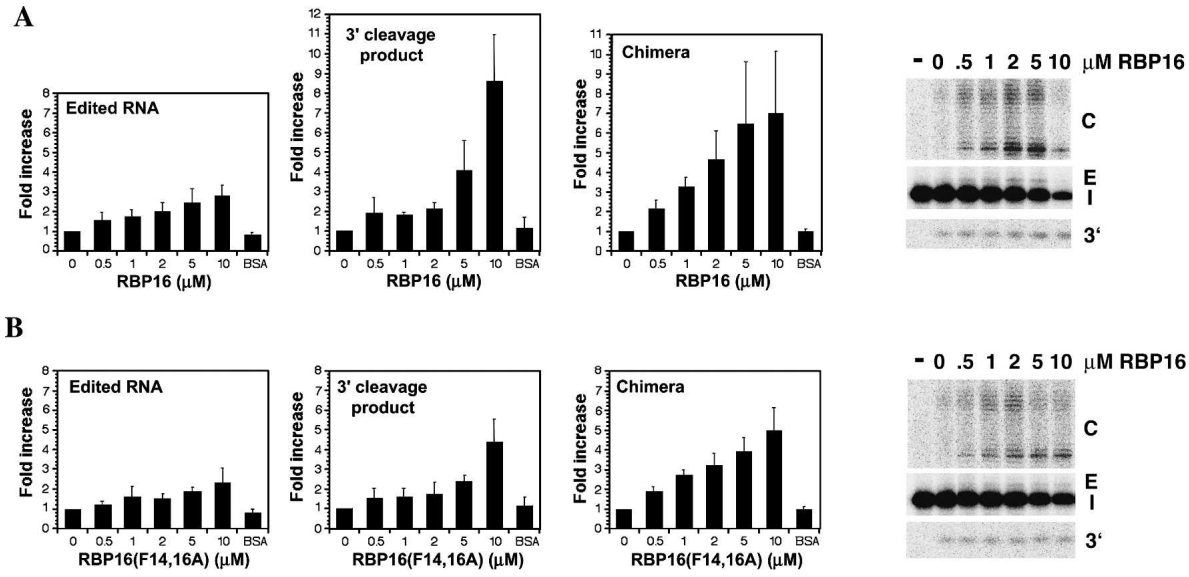

FIGURE 7. Effect of RBP16 or RBP16(F14,16A) on in vitro insertion editing of A6 with natural gRNA. RBP16 (*A*) or RBP16(F14,16A) (*B*) was titrated into in vitro insertion reactions (20 μL final volume) containing [^{32}P]pCp 3′-labeled A6 pre-mRNA (~10 fmol) and 1.25 pmol natural A6 gRNA as described in Materials and Methods. Quantitation of edited RNA, 3′ cleavage products, and chimeras is expressed in graphical form as in Figure 3. Representative experiments, labeled as in Figure 4, are presented on the *right*. (C) chimeras.

(see Fig. 1B). As with the natural A6 gRNA, RBP16 stimulated the formation of edited A6 RNA up to about 2.5-fold (Fig. 8A). Stimulation of 3′ cleavage product formation at the highest RBP16 concentration (10 μM) was reduced with the enhanced gRNA compared to the natural A6 gRNA (cf. Figs. 7A and 8A, 3′ cleavage product). No chimera formation was detected with this pre-mRNA–gRNA pair, presumably due to a more stable mRNA–gRNA association compared to the natural A6 gRNA. Comparison of the effect of RBP16 versus RBP16(F14,16A) on edited RNA or 3′ cleavage product formation revealed essentially no difference between mutant and wild-type RBP16 (Fig. 8, cf. A and B). Since there was no difference in the ability of RBP16 or RBP16(F14,16A) to stimulate editing using the enhanced A6 gRNA (Fig. 8), while differences were observed with the natural gRNA (Fig. 7), this suggests that binding by the CSD of a gRNA element that is different between the two gRNAs is a component of the enhancement of in vitro insertion editing by RBP16. Because the enhanced A6 gRNA lacks an extended oligo(U) tail, these results point to an effect of CSD–oligo(U) tail binding as one component of editing enhancement by RBP16.

DISCUSSION

We report here experiments aimed at elucidating the mechanism of RBP16 function in RNA editing. Using in vitro RNA editing assays, we demonstrate that recombinant RBP16 is able to significantly stimulate in vitro insertion editing of both CYb and A6 pre-mRNAs. Formation of 3′ cleavage product and mRNA–gRNA chimeras was also enhanced. Stimulation of CYb RNA editing in vitro by RBP16 is consistent with our previous data showing that RNAi-mediated down-regulation of RBP16 results in an almost complete loss of CYb editing in vivo. Thus, the data presented here support the definition of RBP16 as an RNA editing accessory factor in *T. brucei*.

Our results suggest that the mechanism by which RBP16 stimulates RNA editing involves molecular interactions with both the CSD and RGG RNA binding domains of the protein. We previously showed that a large proportion of RBP16 gRNA binding affinity as well as its specificity for the gRNA oligo(U) tail can be attributed to interactions involving the protein's N-terminal CSD (Miller and Read 2003). To determine the contribution of RNA binding by the CSD in editing stimulation, we introduced mutations that essentially abolished the RNA binding activity of that domain, creating RBP16(F14,16A). Surprisingly, the RBP16 CSD mutant was still able to stimulate editing, and, at high concentrations of the protein, stimulation was comparable to levels observed with the wild-type protein. This finding is reminiscent of the ability of high concentrations of SR proteins lacking an RNA binding domain to facilitate in vitro pre-mRNA splicing at levels similar to intact protein (Shen and Green 2004). In the latter study, it was proposed that the sole function of the RNA binding domain is to position the RS domain in the vicinity of the splicing signal, rendering the RNA binding domain dispensable in the presence of a high concentration of the RS domain. The CSD and RGG domains of RBP16 may be playing similar relative roles during in vitro RNA editing. In any case, the capacity of RBP16(F14,16A) to promote substantial levels of in vitro editing strongly suggests that the weaker and less specific RGG domain–RNA interactions are important in

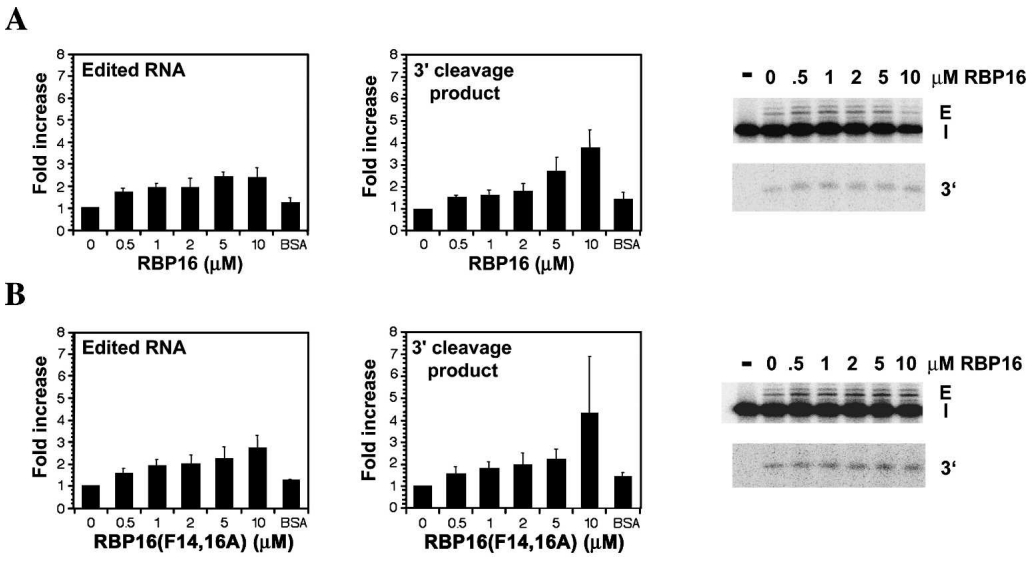

FIGURE 8. Effect of RBP16 or RBP16(F14,16A) on in vitro insertion editing of A6 with enhanced gRNA. RBP16 (*A*) or RBP16(F14,16A) (*B*) was titrated into in vitro insertion reactions as described in Figure 7. Quantitation of edited products and 3′ cleavage products is expressed in graphical form as in Figure 3. Representative experiments, labeled as in Figure 4, are presented on the *right*.

the action of RBP16 during editing. Previous results implicated the RBP16 RGG domain in facilitating multiple RNA conformations (Miller and Read 2003). Together, these results suggest that modulation of RNA structure by RBP16 might be important for enhancing the efficiency of certain RNA editing events. While the RNA binding capacity of the CSD is not strictly required for editing stimulation, two lines of evidence support a role for CSD–RNA binding in RBP16 enhancement of editing. First, we consistently observed that stimulation of edited RNA production by RBP16(F14,16A) was nonsaturable in contrast to the saturable nature of the reaction with wild-type RBP16. A nonsaturable reaction suggests the contribution of nonspecific, low affinity protein–RNA contacts that prevent RBP16 from saturating the RNA substrate. This type of binding would be consistent with the proposed mode of interaction of the RBP16 RGG domain with the encoded portion of the gRNA (Miller and Read 2003). Hence, the saturable nature of editing stimulation by wild-type RBP16 suggests that CSD–RNA interactions are contributing to this reaction. The importance of the CSD was further supported by experiments with the A6 editing system, using enhanced or natural gRNAs. The ability of the RBP16 (F14,16A) mutant to stimulate editing of the A6 pre-mRNA was only reduced compared to wild-type RBP16 when a natural gRNA containing an extended oligo(U) tail, and with less extensive base-pairing with the pre-mRNA, was used. These results support an effect of CSD–oligo(U) tail binding in RBP16 function during in vitro editing. Overall, our results lead us to conclude that the RNA binding capacities of both the CSD and RGG domains are important for maximal effect of RBP16 stimulation of RNA editing.

RBP16 stimulates in vitro editing either at, or prior to, endonucleolytic cleavage of pre-mRNA, as shown by the RBP16-mediated enhancement of pre-mRNA 3′ cleavage product accumulation under all assay conditions tested. We also observed an increase in chimera production using natural A6 and CYb gRNAs (Fig. 7; data not shown). The same machinery is responsible for both faithful editing and creation of chimeric byproducts, and the pathways leading to creation of these two products diverge post-cleavage. Thus, stimulation of chimera production by RBP16 is indicative of RBP16 function at an early step common to full editing and production of chimeras. The demonstration that RBP16 acts at an early step in the editing cycle suggests several potential mechanisms of action. RBP16–RNA interactions may play an important role in this effect. For example, RBP16 may act prior to endonucleolytic cleavage by promoting association of gRNA and/or mRNA with the editing machinery. Alternatively, RBP16 may possess RNA annealing activity that promotes the gRNA–mRNA interaction, similar to the reported annealing activity of the related CSD-containing protein, YB-1 (Skabkin et al. 2001). The MRP1/2 complex and its MRP1 constituent, which are the only other genetically implicated RNA editing accessory factors (Vondrusková et al. 2005), have been shown to promote gRNA–mRNA annealing in vitro (Müller et al. 2001; Müller and Göringer 2002; Aphasizhev et al. 2003a). However, it is important to note that RBP16 and MRP1/2 do not perform redundant functions, as both are essential for CYb RNA editing in vivo (Pelletier and Read 2003; Vondrusková et al. 2005). RBP16–RNA interactions may also effect proper positioning of RNAs within the editosome environment such that they are efficiently presented to endonuclease active sites. We are currently testing these possibilities both in vitro and in vivo.

In addition to potential RNA mediated effects, we cannot rule out the possibility that RBP16 stimulates pre-mRNA cleavage through a direct protein–protein interaction. RBP16 has not been detected as a stable component of any editing complex preparation obtained by either chromatography or immunoprecipitation (Panigrahi et al. 2001, 2003a,b; Schnaufer et al. 2003). However, as an editing accessory factor, RBP16 would be expected to interact only transiently with the editosome and therefore be present in substoichiometric amounts. A transient association with the editing machinery would limit the likelihood of detecting RBP16 interaction with editosome components, as well as its presence within a core editosome preparation. Enhancement of pre-mRNA cleavage by RBP16 may indicate that the protein interacts with an endonuclease. The KREPA3 editosome component has been shown to possess endonuclease activity in vitro (Brecht et al. 2005), and specific insertion and deletion editing endonucleases have recently been identified (Carnes et al. 2005; Trotter et al. 2005). It would be of interest to assess whether RBP16 has the ability to enhance the activities of any of these proteins. Finally, our results do not rule out that RBP16 also affects later steps of the editing pathways. Direct effects of RBP16 on specific points of the editing cycle will be tested in future studies.

We observed that the strict specificity of RBP16 for regulation of CYb RNA editing in vivo is not maintained in vitro. Although A6 editing is entirely insensitive to RBP16 depletion in vivo, RBP16 is able to stimulate A6 RNA editing in vitro, albeit to lower levels than CYb editing. These results suggest that RBP16 may be able to perform a function during in vitro A6 editing that is normally carried out by a different editing accessory factor in vivo. Alternatively, the specificity of editing accessory proteins in vivo may be partially conferred by protein binding partners. For example, RBP16-interacting proteins may promote interaction with CYb RNAs or preclude interaction with A6 RNAs. RBP16 is expressed in both PF and bloodstream form (BF) stages of the *T. brucei* life cycle (Pelletier and Read 2003). Since CYb RNA is edited exclusively in PF, it will be interesting to determine whether RBP16 down-regulation in BF trypanosomes affects BF-specific editing events that are normally regulated by

virtue of RBP16 association with BF specificity factors. In addition, in vitro editing assays performed using BF extracts (Halbig et al. 2004) may provide insight into the life-cycle-stage-specific functions of RBP16. We are currently identifying RBP16-interacting proteins using yeast two-hybrid screens and TAP-tagging approaches. Proteins that confer the specificity of RBP16 for certain RNAs in different life cycle stages may be identified during these studies. Finally, the somewhat increased ability of RBP16 to enhance CYb editing compared to A6 editing in vitro also suggests the possibility that CYb RNAs possess intrinsic sensitivity to RBP16 stimulation, which may be greatly increased in vivo in the presence of full-length, native pre-mRNA and gRNA.

In summary, these results represent the first example of an RNA editing accessory factor capable of directly stimulating trypanosome RNA editing in vitro. They further suggest mechanisms by which RBP16 may affect RNA editing in vivo, by demonstrating that the protein stimulates editing at an early step in the editing reaction and indicating an important role for the RGG domain of the protein.

MATERIALS AND METHODS

Preparation and labeling of RNAs

CYb pre-mRNA insertion was assayed at ES1 using either the natural gCYb[558] gRNA (+2U) (Riley et al. 1994) or the enhanced gRNA gCYb[558]USD-2A (+2U) with pre-mRNA CYb Anchor as described previously (Igo et al. 2002). ATPase 6 (A6) pre-mRNA insertion was assayed at ES2 using the natural gRNA g[2,4] (+2U) (Cruz-Reyes et al. 1998) or the enhanced gRNA gA6[14]USD-3A (+3U) (Igo et al. 2002) with pre-mRNA m[0,4] (Cruz-Reyes and Sollner-Webb 1996) (previously termed A6short; Seiwert et al. 1996). RNAs utilized in editing assays were synthesized in vitro using an Ambion T7 Megascript kit and purified by gel electrophoresis on 6% acrylamide/7 M urea. Radiolabeling of pre-mRNA at the 3′ end was performed by ligation of [5′-^{32}P]pCp (Perkin Elmer) by T4 RNA ligase (Promega). RNAs used in gel retardation assays were synthesized and internally radiolabeled with [α-^{32}P] UTP using an Ambion T7 Maxiscript kit as described (Miller and Read 2003) and were identical in sequence to those used in editing assays.

Protein expression and purification

C-terminal His-tagged RBP16 was produced as previously described (Hayman et al. 2001). His-tagged RBP16(F14,16A) was constructed by PCR amplification from a pET-21a plasmid encoding the mature His-RBP16 using the QuikChange multi site-directed mutagenesis kit (Stratagene) and the mutagenic primer RBP16F14A,F16A (5′-GATGTCTGGACGTGGTGCTGGTGCTATTGAAGACGACGC-3′). Protein induction and nickel affinity chromatography were as described previously for His-RBP16 (Hayman et al. 2001). RBP16(F14,16A) was further purified by Q-Sepharose chromatography after dialysis in 20 mM Tris (pH 8.7), 25 mM KCl. Upon elution with a step gradient of increasing KCl, the purified protein was collected in the 50 mM KCl elution fraction. All proteins were dialyzed in buffer B (Miller and Read 2003) upon elution and concentrated using a Centricon-10 microconcentrator (Amicon). The purity and integrity of expressed proteins were examined by silver stain of 15% SDS-PAGE gels. Protein concentrations were determined using the Bio-Rad protein assay with bovine serum albumin (BSA) standards.

Cells, extract preparation, and extract fractionation

Procyclic form (PF) *T. brucei brucei* clone IsTaR1 stock EATRO 164 was grown as described (Brun and Schonenberger 1979). Mitochondrial extract (~2×10^{10} cell equivalents/mL) was prepared and editing complex was purified by Q-Sepharose and DNA cellulose chromatography as described (Rusché et al. 1997) with the following modifications. Mitochondrial extract was supplemented with complete protease inhibitors (Roche, EDTA-free) rather than individual protease inhibitors, and an additional Q-Sepharose purification step identical to the first was performed following DNA cellulose chromatography to increase the stringency of the isolation procedure (Sollner-Webb et al. 2001). Fractions from the final step of these purification procedures were tested for insertion editing activity, and peak-activity fractions were used for the experiments.

Gel retardation assays

Reaction conditions were as described previously (Read et al. 1994). Increasing amounts (0.5–10 μM) of RBP16 or RBP16 (F14,16A) were incubated with 5 fmol or 2 fmol of internally radiolabeled CYb or A6 RNAs, respectively, for 20 min at room temperature. Protein–RNA complexes were separated by electrophoresis on native 8% acrylamide gels (acrylamide/bisacrylamide ratio 19:1) in 50 mM Tris-glycine (pH 8.8). Shifted bands were detected by autoradiography.

UV cross-linking assays

Reaction conditions were identical to gel retardation assays, and cross-linking and subsequent RNase digestion were carried out as described previously (Read et al. 1994). Reactions were fractionated by electrophoresis on 15% SDS gels. Labeled proteins were detected by autoradiography.

Editing reactions

CYb insertion reactions were performed in a final volume of 20 μL containing 10 mM KCl-MRB buffer (Piller et al. 1995) (25 mM Tris-Cl at pH 8.0, 10 mM $Mg(OAc)_2$, 10 mM KCl, 1 mM EDTA at pH 8.0, 0.5 mM DTT, 5% glycerol) supplemented with 0.3 mM ATP (Amersham), 150 μM UTP (Amersham), 5 mM $CaCl_2$, 25 ng/μL acetylated BSA (Invitrogen), and 0.1 ng/μL torula RNA (Sigma). Reactions utilized [^{32}P]pCp 3′-labeled pre-mRNA (~10 fmol), 1.25 pmol gRNA, and 2 μL final Q-Sepharose purified fraction. A6 insertion reactions were identical to CYb reactions, except that ATP was reduced to 0.3 μM and torula RNA was omitted.

In most cases, reaction components, including RBP16, were combined and incubated at room temperature for 5 min prior to addition of editing complex. After addition of editing complex,

reactions were continued for 1 h at 26°C. Alternatively, for reactions in which RBP16 was preincubated with a specific reaction component (gRNA or mRNA), RBP16 and the reaction component were combined in a volume of 3 μL (~7× final concentration) and incubated for 5 min at room temperature. The remaining reaction components (minus editing complex) were then added and incubated an additional 5 min at room temperature as in the no preincubation reactions. Acetylated BSA (150 ng/μL) was included as a negative control and represents an approximately equal mass amount of protein relative to the highest concentration of RBP16 tested (10 μM). As an additional negative control, PEG 3350 (Sigma) (2% or 4%), a molecular crowding agent, was titrated into in vitro insertion reactions to control for nonspecific effects on the assay due to molecular crowding as a result of adding exogenous protein.

Upon completion, all reactions were extracted with phenol-chloroform and precipitated prior to resolving on 60-cm-long 9% polyacrylamide/ 8 M urea gels. Quantitation of edited RNAs, 3′ cleavage products, and chimeras was performed using a Personal Molecular Imager FX phosphorimager (Bio-Rad). Data are expressed as the fraction of input RNA converted to each product normalized to the amount obtained in the absence of additional protein, hence fold increase over no protein levels. Results from three independent experiments are displayed as mean and standard deviation.

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