Differential Effects of Arginine Methylation on RBP16 mRNA Binding, Guide RNA (gRNA) Binding, and gRNA-containing Ribonucleoprotein Complex (gRNP) Formation*

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Mitochondrial gene expression in *Trypanosoma brucei* involves the coordination of multiple events including polycistronic transcript cleavage, polyadenylation, RNA stability, and RNA editing. Arg methylation of RNA binding proteins has the potential to influence many of these processes via regulation of protein-protein and protein-RNA interactions. Here we demonstrate that Arg methylation differentially regulates the RNA binding capacity and macromolecular interactions of the mitochondrial gene regulatory protein, RBP16. We show that, in *T. brucei* mitochondria, RBP16 forms two major stable complexes: a 5 S multiprotein complex and an 11 S complex consisting of the 5 S complex associated with guide RNA (gRNA). Expression of a non-methylatable RBP16 mutant protein demonstrates that Arg methylation of RBP16 is required to maintain the protein-protein interactions necessary for assembly and/or stability of both complexes. Down-regulation of the major trypanosome type 1 protein arginine methyltransferase, TbPRMT1, disrupts formation of both the 5 and 11 S complexes, indicating that TbPRMT1-catalyzed methylation of RBP16 Arg-78 and Arg-85 is critical for complex formation. We also show that Arg methylation decreases the capacity of RBP16 to associate with gRNA. This is not a general effect on RBP16 RNA binding, however, since methylation conversely increases the association of the protein with mRNA. Thus, TbPRMT1-catalyzed Arg methylation has distinct effects on RBP16 gRNA and mRNA association and gRNA-containing ribonucleoprotein complex (gRNP) formation.

Gene regulation in kinetoplastid protozoa such as trypanosomes and *Leishmania* is effected primarily at the post-transcriptional level (1). In the mitochondria of *Trypanosoma brucei*, gene expression involves the coordination of multiple processes including ribonucleolytic processing of primary transcripts, polyadenylation, RNA stability, and RNA editing (2–5).[2] RNA editing is required for the creation of translatable mRNAs from 12 of the 18 mitochondrial protein-coding genes. Kinetoplastid editing entails insertion and deletion of uridine residues at numerous editing sites as directed by mitochondrially encoded, *trans*-acting guide RNAs (gRNAs).[3] During editing, the gRNA 5' anchor region base pairs with the target mRNA 3' of the editing site. An internal information coding region, present within the gRNA, guides uridine insertion and deletion through base pairing. A post-transcriptionally added poly(U) tail, present on the 3' end of all mature gRNAs, provides stabilization of mRNA-gRNA interactions. This transcript maturation progresses in a 3'-5' direction and is catalyzed by a large multisubunit complex termed the editosome (4, 6–8). The enzymatic reactions involved in editing include endonucleolytic cleavage of the mRNA, uridine insertion or deletion, and religation of the 3' and 5' mRNA fragments. Although the core components of the editosome are under intense study, accessory proteins that augment and/or regulate editing have only just begun to be identified (9, 10).

The RNA-binding protein, RBP16, has been implicated in a variety of processes within the mitochondria of *T. brucei*, including RNA editing and stability (9, 11, 12). In accordance with its multifunctional nature, RBP16 is classified as a member of the Y-box family, which includes proteins that perform multiple roles in gene expression. For example, the mammalian Y-box protein, YB-1, acts in transcription, RNA stability, translation, and RNA packaging (13–17). RBP16 function has been examined by both targeted depletion and over expression studies. Cells depleted for RBP16 via RNA interference (RNAi) display severe defects in growth as well as a dramatic and specific down-regulation in the editing of the mitochondrial apocytochrome b (CYb) transcript (9). Consistent with this finding, CYb RNA editing is stimulated *in vitro* by addition of recombinant RBP16 at, or prior to, the endonucleolytic cleavage of mRNA (12). RBP16 also plays a complex role in RNA stabilization in *T. brucei* mitochondria. The stability of the never-edited mitochondrial transcripts cytochrome oxidase I (COI) and NADH dehydrogenase subunit 4 (ND4) is decreased upon RBP16 down-regulation (9), while overexpression of RBP16

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[2] J. L. Mattiacio and L. K. Read, unpublished results.

[3] The abbreviations used are: gRNA, guide RNAs; RNAi, RNA interference; CYb, apocytochrome b; PRMT, protein arginine methyltransferase; ND4, NADH dehydrogenase subunit 4; COI, cytochrome oxidase I; RNP, ribonucleoprotein; gRNP, gRNA-containing RNP complex; CAPS, (cyclohexylamino)-1-propanesulfonic acid; WT, wild-type; TRI, triple mutant; A6, ATPase subunit 6.

serves to stabilize edited and unedited CYb and cytochrome oxidase III mRNAs (11).

In mitochondria, RBP16 is associated with ~30% of the total gRNA pool, 9 S and 12 S rRNAs, and several mitochondrial mRNA species, as demonstrated by *in organello* cross-linking and co-immunoprecipitation from mitochondrial extract (18, 19).[4] The CYb transcript, whose editing is RBP16-dependent, is minimally edited and thus presumably utilizes a very small percentage of the total gRNA (20). The disparity between the percentage of gRNAs involved in CYb editing and the large percentage of the total gRNA population that is bound by RBP16 *in vivo* suggests an additional function for RBP16 relating to the packaging and protection of gRNA and/or the delineation of their usage. Furthermore, the transcript-specific facets of RBP16 action in combination with its broad RNA binding properties strongly argues that RBP16 functions in association with additional mitochondrial proteins, some of which may confer sequence specificity (9, 19, 21, 22).

RBP16 is post-translationally modified by arginine methylation on three residues in its RG-rich C terminus. Genetic experiments demonstrated that methylation is important for many of the RNA stabilization functions of RBP16 (11). In yeast and mammals, Arg methylation has been associated with a variety of cellular processes including signal transduction, subcellular localization, transcription, and RNA processing (23–27). The effects of Arg methylation on these processes are mediated primarily through the regulation of protein-protein and, less commonly, protein-RNA interactions (28–31). Arg methylation has also been recognized as a targeting signal in the process of ribonucleoprotein (RNP) complex assembly (32, 33). The role of Arg methylation in RBP16 protein-protein and protein-RNA interactions has not been explored at the biochemical level.

In this paper, we demonstrate that native RBP16 is almost entirely present in two macromolecular complexes of 5 and 11 S, the larger of which is a gRNA-containing ribonucleoprotein complex (gRNP). The 11 S gRNP is reduced to a 5 S complex upon RNase treatment. These data, along with the inability to identify gRNA in the endogenous 5 S complex, indicate that the 5 S complex is a protein subunit of the 11 S gRNP. Exogenously expressed non-methylatable RBP16 is compromised for 5 and 11 S complex formation, in a similar fashion as endogenous RBP16 in cells down-regulated for the major trypanosome type 1 protein arginine methyltransferase (PRMT), TbPRMT1. Thus, Arg methylation of RBP16 is necessary for the proper macromolecular interactions involved in formation of both endogenous complexes. Our data are consistent with a model in which Arg methylation is required for maintenance of RBP16 protein-protein interactions. We also show that, in contrast to its role in facilitating RBP16 protein-protein interactions, Arg methylation significantly decreases the ability of RBP16 to associate with gRNA. This does not reflect a general inhibition of RBP16-RNA interactions, however, since methylation strongly increases the association of RBP16 with specific mRNAs. Together, our data demonstrate distinct roles for RBP16 methylation in specific protein-RNA and protein-protein interac-

tions. Thus, Arg methylation of RBP16 has the potential to regulate the actions of this multifunctional protein.

EXPERIMENTAL PROCEDURES

DNA Oligonucleotides—For poisoned primer extension analyses, the following oligodeoxynucleotides were used: A6-3′ NE (5′-GCGGATCCATTTGATCTTATTCTATAACTCC-3′), CYb-RT-GP (5′-CAACCTGACATTAAAAGAC-3′), COI-RT (5′-GTAATGAGTACGTTGTAAAACTG-3′), ND4-RT (5′-GAT-AAAAAATATTAGTGACATTG-3′).

Trypanosome Growth, Transfection, and Induction of Myc-RBP16—Procyclic *T. brucei* strain 29-13 (provided by George Cross), which contains integrated genes for T7 RNA polymerase and the tetracycline repressor, was grown in SDM-79 supplemented with 15% fetal bovine serum as described (34, 35), in the presence of G418 (15 μg/ml) and hygromycin (50 μg/ml). The Myc-RBP16-WT, Myc-RBP16-TRI (11), and TbPRMT1 RNAi cell lines (36) were supplemented with phleomycin (2.5 μg/ml). Exogenous protein expression and RNAi was induced by adding 2.5 μg/ml tetracycline and allowing the cultures to grow for 4 days prior to mitochondrial harvesting (11). Mitochondria were purified as described (37).

Glycerol Gradient Sedimentation—Mitochondrial extract was obtained from 1×10^{10} cell equivalents by adding 500 μl of mitochondrial lysis buffer (25 mM Tris-HCl (pH 8.0), 50 mM KCl, 10 mM MgOAc, 100 μM ATP, 1% glycerol, 0.2% Nonidet P-40, Complete® EDTA-free protease inhibitors (Roche Diagnostics) and 40 units of RNaseOUT (Invitrogen) (note: RNaseOUT not added to samples to be RNase-treated)) to purified mitochondria and rocking for 5 min at 4 °C prior to clarification by centrifugation at 13,000 rpm for 15 min. Samples that were RNase A-treated were incubated with 100 μg/ml RNase A (Sigma) at 37 °C for 15 min prior to loading onto gradients. His-RBP16 was purified as described previously (38). Five-hundred μl of purified mitochondrial extract or 1.6 μg of recombinant His-RBP16 was layered onto a 12-ml 5–20% linear glycerol gradient (25 mM Tris-HCl (pH 8.0) 50 mM KCl, 10 mM MgOAc, 100 μM ATP, Complete® EDTA-free protease inhibitors (Roche Diagnostics) and 5 or 20% glycerol). The gradient was centrifuged for 20 h at 4 °C in a Beckman SW-41 rotor at 35,000 rpm. Twenty-four fractions (500 μl) were collected from the top of the tube.

Western Blotting—For analysis of 29-13, Myc-RBP16-WT, and Myc-RBP16-TRI extracts fractionated by glycerol gradient sedimentation, 10-μl aliquots of each fraction were separated by 17% SDS-PAGE and transferred electrophoretically to a nitrocellulose membrane (Bio-Rad) at 50 V for 35 min in 10 mM CAPS buffer (pH 11.0) containing 10% methanol. The membrane was probed using either polyclonal anti-RBP16 (21) or polyclonal anti-Myc (Covance) antibodies at a 1:1000 dilution in Tris-buffered saline with 2% dry milk. Primary antibodies were detected using goat anti-rabbit antibody coupled to horseradish peroxidase (Pierce Endogen), and detected by ECL (GE Healthcare).

Immunoprecipitation and Isolation of Associated RNA—Ten μg of purified anti-RBP16 (21), anti-Myc (Covance), or IgG purified from pre-immune serum was incubated with 250 μl of

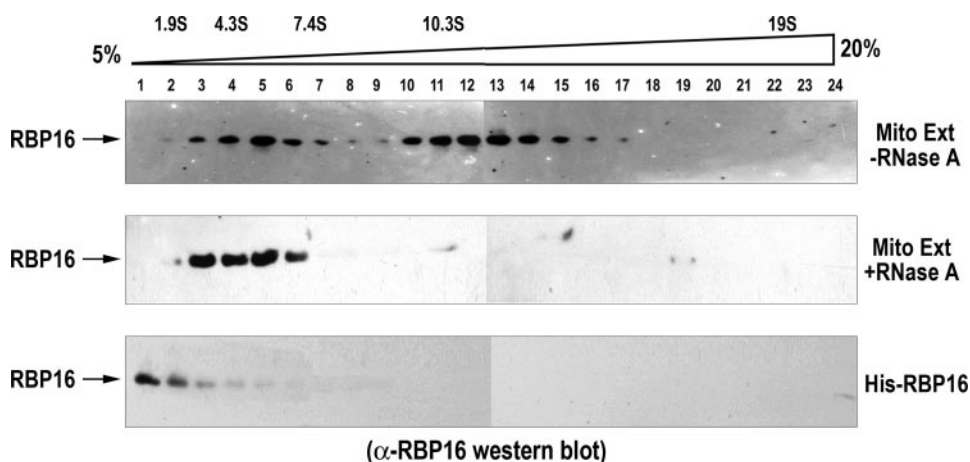---

[4] M. Pelletier and L. K. Read, unpublished results.

FIGURE 1. RBP16 forms two macromolecular complexes of 5 and 11 S. Mitochondrial extract from strain 29-13 procyclic *T. brucei* was sedimented on a 5–20% glycerol gradient and fractionated into 24 aliquots. Fractions were separated by SDS-PAGE on a 17% gel and probed by anti-RBP16 Western blot. Sedimentation coefficients were determined by sedimentation of markers in a parallel gradient. The *top panel* corresponds to untreated mitochondrial extract, the *middle panel* is mitochondrial extract pretreated with RNase A prior to sedimentation, and the *bottom panel* represents sedimentation of recombinant His-RBP16 alone.

mitochondrial extract or 500 μl of pooled gradient fractions with rocking at 4 °C for 1 to 2 h. Twenty μl of rProtA-Sepharose (50% slurry) (GE Healthcare) washed twice in mitochondrial lysis buffer (see above) was added to each tube and incubated with rocking at 4 °C for 1 to 2 h. The bead bound material was pelleted by centrifugation at 13,000 rpm for 5 min and the non-bound supernatant transferred to a separate tube. The beads were washed three times with 1 ml of mitochondrial lysis buffer and resuspended in a volume of mitochondrial lysis buffer equivalent to the non-bound supernatant. For protein analysis, 10% of each fraction was mixed with an appropriate volume of 5× SDS-PAGE sample buffer (500 mM Tris-HCl (pH 8.0), 10% SDS, 50% glycerol, 0.04% bromphenol blue, and 400 mM dithiothreitol). For RNA analysis, the remainder of each fraction was incubated with 0.5% SDS (final), 50 μg/ml of proteinase K (final) (Promega), and 2 units of RNaseOut. After 30 min at 37 °C, RNA was twice extracted with phenol/chloroform and salt/ethanol-precipitated. Immunoprecipitation of total mitochondrial extract and 5 and 11 S complexes, as well as subsequent bound RNA analysis (see below), was performed three to four times, using at least two different mitochondrial preparations, with similar results.

Guanylyltransferase Labeling—An aliquot of each RNA sample was labeled by incubation with 10 μCi of α-[^{32}P]GTP and 2.2 pmol of recombinant guanylyltransferase from vaccinia virus (a generous gift of Ed Niles) (39, 40) for 30 min at room temperature in 50 mM Tris-HCl (pH 8.0), 1.5 mM MgCl$_2$, 6 mM KCl, 2.5 mM dithiothreitol, and 40 units of RNaseOUT. An equal volume of 90% formamide loading buffer was added to each sample and one third of each reaction was separated on a 7 M urea/10% polyacrylamide gel in 0.6× TBE. Gels were analyzed by overnight exposure to a Bio-Rad Phosphor Imaging Screen K and scanning by Bio-Rad Personal FX with densitometry analysis using Bio-Rad Quantity One software. Differential RNA species were identified based on previously published sizes (21, 41, 42) as compared with labeled markers. The percent bound for each lane was calculated by adding the relative abundance of the bound and not bound lanes and dividing by

the sum. The amount specifically bound was calculated by subtracting the percent bound by the preserum from the percent bound by the purified antibody.

Poisoned Primer Extension Analyses—Poisoned primer extensions were performed essentially as described (11), except using immunoprecipitated RNA and 0.5 pmol of 5′-^{32}P-labeled oligodeoxynucleotide primer.

His-RBP16/gRNA Binding Assay—The gCYb[558] clone was described previously (20). RNA was synthesized *in vitro* using the Ambion Megascript kit and gel-purified on a 7 M urea, 6% acrylamide gel. One-hundred fmol (1.6 μg) of purified His-RBP16 (38) was incubated alone or with either 100 fmol (2.2 μg) of gCYb[558] (1:1 molar ratio) or 1 nmol (22 μg) of gCYb[558] (10:1 molar ratio) at 27 °C for 20 min in mitochondrial lysis buffer prior to glycerol gradient fractionation.

RESULTS

RBP16 Is Present in Two Complexes of 5 and 11 S—To identify RBP16 macromolecular interactions we analyzed the ability of RBP16 to form multicomponent complexes by glycerol gradient sedimentation. Mitochondrial extract from procyclic strain 29-13 *T. brucei* cells was fractionated on a linear 5–20% gradient. Analysis of gradient fractions by anti-RBP16 Western blot revealed two RBP16-containing complexes of ~5 and 11 S, based on sedimentation of markers in a parallel gradient (Fig. 1, *top*). In contrast, recombinant His-RBP16 displayed a sedimentation coefficient of ~1.2 S (Fig. 1, *bottom*), confirming previous results that free RBP16 does not form homomultimers (18, 43). Thus, the majority of native RBP16 is present in complexes with other macromolecules.

To determine whether the 5 S and/or 11 S complex contains an RNA component, we pretreated mitochondrial extract with RNase A for 15 min at 37 °C prior to gradient fractionation. As shown in Fig. 1 (*middle*), the 11 S complex was completely disrupted following RNase treatment, while the 5 S complex was apparently unaffected. These data indicate that the 11 S complex contains an unprotected RNA component, while the 5 S complex is either lacking an RNA component or contains an RNA that is protected from RNase A treatment.

The RBP16 11 S Complex Contains gRNA—Because RBP16 has previously been shown to bind ~30% of the total gRNA pool *in vivo* (9, 21), we wanted to determine whether the RNA component of the major RBP16-containing complexes was gRNA. We first asked whether a substantial portion of total mitochondrial gRNA co-sediments with either the 5 or 11 S complex. To this end, RNA from each fraction of a 5 to 20% glycerol gradient was extracted and labeled with α-[^{32}P]GTP using recombinant guanylyltransferase from vaccinia virus (40). This procedure only labels primary transcripts that pos-

A

B

55%   45%   62%   38%
(0% +/- 3% spec. bound)

84%   16%   63%   37%   (% bound)
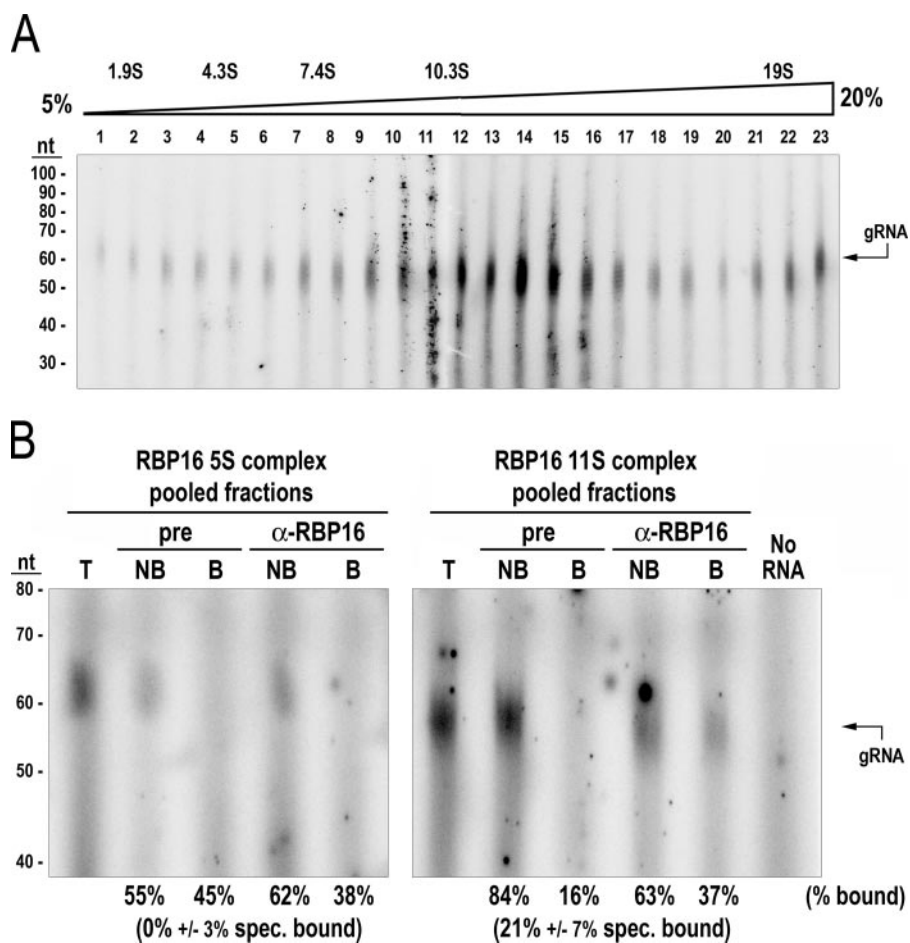(21% +/- 7% spec. bound)

FIGURE 2. **The 11 S complex contains gRNA.** *A*, total RNA from each of the glycerol gradient fractions from Fig. 1 was isolated by ethanol/salt precipitation and labeled with α-[^{32}P]GTP using recombinant guanylyltransferase, which specifically labels gRNA. Radiolabeled products were separated on an 7 M urea, 10% acrylamide gel and visualized by phosphorimager analysis. *B*, peak RBP16-containing glycerol gradient fractions (3–6 for 5 S and 11–14 for 11 S) were pooled and immunoprecipitated with preimmune IgG or anti-RBP16 antibodies, and the associated RNA was isolated. RNA was labeled with guanylyltransferase as described for A. The *left panel* shows RNA present in the total (*T*) and immunoprecipitated 5 S region of the gradient, while the *right panel* corresponds to the 11 S region of the gradient. *Percent bound* and *percent specifically bound* were calculated as described under "Experimental Procedures." The images shown are representative of the results. The values under each lane are the average quantification of the percent bound from three experiments. The percent of specifically bound gRNA in each complex is shown in *parentheses* below the figures and represents the average and standard deviation from three experiments. *T*, total extract; *NB*, not bound; *B*, bound; *pre*, purified IgG from pre-immune serum; α-*RBP16*, affinity-purified anti-RBP16.

in anti-RBP16 immunoprecipitates from these regions of the gradient revealed that an average of 21% of the gRNA in the 11 S region of the gradient is specifically associated with RBP16 (Fig. 2*B*, *right panel*), while the 5 S RBP16-containing complex contains little more than background levels of gRNA (Fig. 2*B*, *left panel*) (for calculation of specifically bound RNA, see "Experimental Procedures"). Together, these data suggest that the 5 S complex is devoid of RNA and consists of RBP16 associated with at least one other protein, while the 11 S complex contains both gRNA and additional protein(s).

Non-methylated RBP16 Fails to Form Endogenous Complexes—RBP16 is methylated on three arginine residues in its C-terminal RG-rich domain (Arg-78, Arg-85, Arg-93) (11, 45). We next asked whether arginine methylation of RBP16 affects assembly of this protein into macromolecular complexes. We previously reported the generation of two cell lines that exogenously express C-terminally Myc-tagged versions of RBP16 (11). The first cell line inducibly expresses wild-type (WT) RBP16, while the second inducibly expresses a non-methylatable triple mutant (TRI) in which the three known methylated arginine residues have been converted to lysine. The Myc-RBP16-WT and Myc-RBP16-TRI proteins are both expressed and subsequently enriched in the mitochondria at levels comparable with that of endogenous RBP16 (11). The simultaneous expression of endogenous and exogenous RBP16 provides an internal control for comparison of complex formation between the two proteins. Additionally, anti-Myc antibodies can be used to specifically isolate Myc-tagged RBP16 containing complexes.

Glycerol gradient fractionation of mitochondrial extract from cells expressing Myc-RBP16-WT followed by anti-RBP16 Western blot verified that Myc-RBP16-WT forms the 5 and 11 S complexes in parallel with endogenous RBP16 (Fig. 3*A*, *upper panel*). These results were confirmed by anti-Myc Western blot (data not shown). Myc-RBP16-WT complexes also behaved identically to endogenous RBP16 upon RNase A treatment, with the 11 S complex disrupted and the 5 S complex apparently unaffected (Fig. 3*A*, *lower panel*). To determine the role of Arg methylation in complex formation, we similarly analyzed mitochondria from cells expressing the non-methylatable Myc-
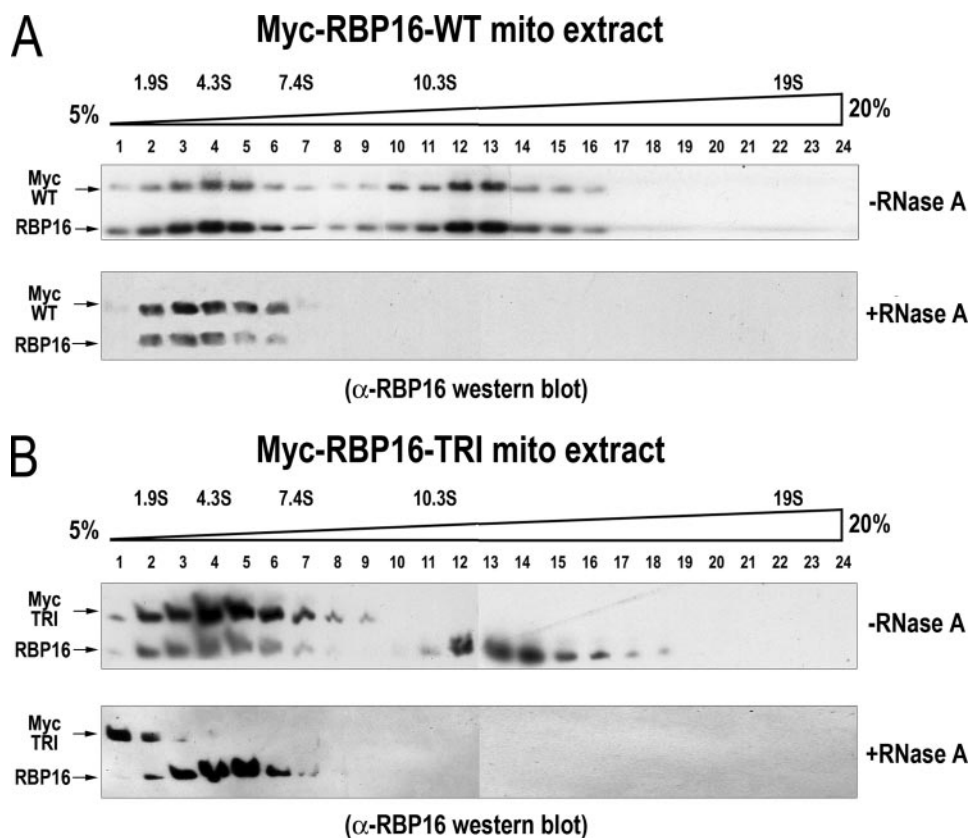
sess a 5' di- or triphosphate (44) and has been previously shown to label primarily gRNAs in kinetoplastid mitochondria (39). As shown in Fig. 2*A*, while gRNAs were broadly distributed across the gradient, the majority of gRNAs sedimented in two peaks of ~11 S and >19 S. The larger peak may represent the association of gRNA with the multiprotein editosome complex. The gRNA peak at 11 S suggests that the 11 S RBP16-containing complex may contain gRNA.

To confirm the presence of gRNA in the RBP16-containing complexes, the peak RBP16 containing fractions (3–6 for 5 S and 11–14 for 11 S) were pooled and immunoprecipitated with anti-RBP16 antibodies, and the associated RNA was extracted. RNA from each fraction was labeled with α-[^{32}P]GTP using recombinant guanylyltransferase as described above (Fig. 2*B*). Examination of the total gRNA from the pooled fractions confirmed that there is significantly more gRNA in the 11 S region than in the 5 S region (Fig. 2*B*, lanes *T*). Analysis of the RNA

A

Myc-RBP16-WT mito extract

B

Myc-RBP16-TRI mito extract

FIGURE 3. **Non-methylatable RBP16 is unable to form endogenous complexes.** *A*, mitochondrial extract from Myc-RBP16-WT cells was sedimented, fractionated, and probed with anti-RBP16 antibodies as described in the legend to Fig. 1. The *upper panel* corresponds to untreated extract, while the *lower panel* represents extract that was pretreated with RNase A prior to sedimentation. *B*, same as described for *A* except using mitochondrial extract from Myc-RBP16-TRI cells.
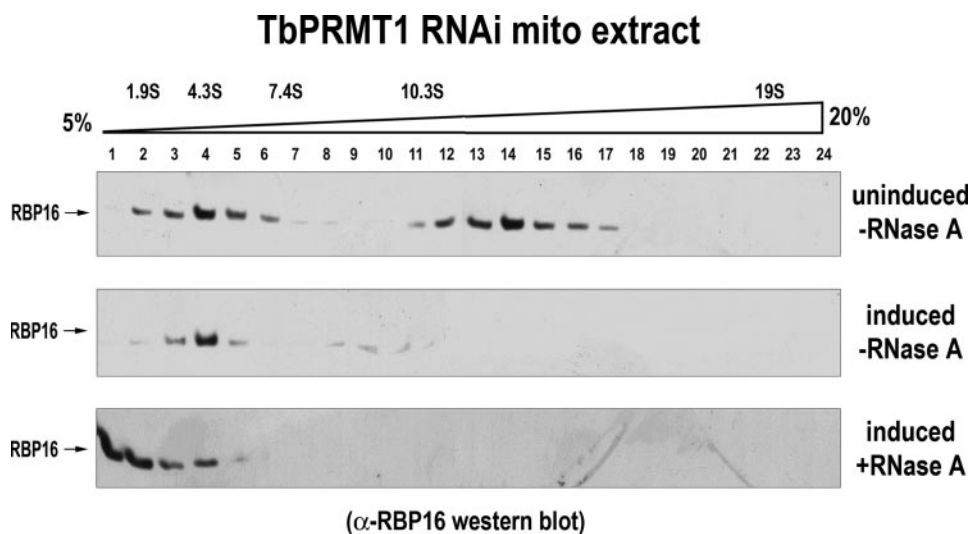
TbPRMT1 RNAi mito extract

FIGURE 4. **RBP16-containing complexes are disrupted in cells depleted for TbPRMT1.** Mitochondrial extract from uninduced and induced (±tetracycline) TbPRMT1 RNAi cells was sedimented and fractionated as described in Fig. 1. The *top panel* corresponds to uninduced extract, the *middle panel* corresponds to untreated induced extract, while the *bottom panel* corresponds to induced extract that was pretreated with RNase A prior to sedimentation.

RBP16 still produced both the 5 and 11 S complexes, demonstrating that the cells remain competent for complex formation. The formation of one complex of ~5 S by Myc-RBP16-TRI is similar to the pattern observed when Myc-RBP16-WT extract is pretreated with RNase A (Fig. 3*A*, *bottom panel*), initially suggesting that the loss of methylation might disrupt protein-RNA interactions. However, when the Myc-RBP16-TRI extract was treated with RNase A prior to fractionation, the 5 S complex was disrupted, leaving only free RBP16 at the top of the gradient (Fig. 3*B*, *bottom panel*). Therefore, the 5 S complex formed with Myc-RBP16-TRI either does not contain the same components as the 5 S complex in the WT cells or methylation serves to protect an RNA component that is exposed to RNase treatment in the complexes containing non-methylated RBP16. From these data, we conclude that Arg methylation of RBP16 is necessary for macromolecular interactions involved in both 5 and 11 S complex formation.

RBP16 Complexes Are Disrupted in Cells Depleted for TbPRMT1— We next wanted to confirm that the differences in complex formation between WT and non-methylatable RBP16 were a result of methylation, or lack thereof, and not amino acid substitution. We previously demonstrated that in cells depleted for the type I PRMT, TbPRMT1, RBP16 Arg-78, and Arg-85 are completely unmethylated, while Arg-93 remains fully methylated (11).

To determine whether methylation of Arg-78 and Arg-85 affects the ability of RBP16 to form macromolecular complexes, mitochondrial extract from cells depleted for TbPRMT1 via tetracycline-inducible RNAi (11, 36) was analyzed by glycerol gradient fractionation. Upon addition of tetracycline to the growth medium, TbPRMT1 RNA was depleted by over 90% (data not shown). Fig. 4 (*top panel*) shows that in uninduced TbPRMT1 RNAi cells, RBP16 forms both the 5 and 11 S complexes, similar to RBP16 in parental 29-13 cells (Fig. 1). However, upon TbPRMT1 depletion, the 11 S complex is almost completely disrupted (Fig. 4, *middle panel*).

RBP16-TRI mutant protein. We found that complex formation by Myc-RBP16-TRI was significantly different from that with endogenous RBP16 or Myc-RBP16-WT. Myc-RBP16-TRI formed only one complex, which sedimented at ~5 S (Fig. 3*B*, *top panel*). In Myc-RBP16-TRI expressing cells, endogenous

Arg Methylation Regulates RBP16 RNA Binding and gRNP Formation

Thus, the stability of the 11 S complex is mediated in part by TbPRMT1, presumably through the methylation of RBP16 and/or other complex components.

When the TbPRMT1-depleted extract was treated with RNase A prior to fractionation, the 5 S complex was disrupted, and RBP16 was present at the top of the gradient (Fig. 4, *bottom panel*), identical to the effects of RNase treatment on RBP16-containing complexes formed with Myc-RBP16-TRI (Fig. 3*B*, *bottom panel*). Thus, TbPRMT1 ablation affects both 5 and 11 S RBP16-containing complexes. While we cannot rule out that these effects are mediated through changes in the methylation status of multiple proteins, we note that the disruption of both RBP16-containing complexes upon TbPRMT1 depletion is strikingly similar to the pattern of complexes formed by Myc-RBP16-TRI. Thus, the simplest explanation for these results is that TbPRMT1-catalyzed methylation of RBP16 is required for proper assembly and/or stability of 5 and 11 S RBP16-containing complexes.

Methylation of RBP16 Negatively Affects Its Association with gRNA—Data presented in Figs. 3 and 4 show that methylation of RBP16 is required for normal protein-RNA interactions. To determine the specific effects of methylation on RBP16-RNA interactions, we quantified the gRNA associated with Myc-RBP16-WT and Myc-RBP16-TRI. We first analyzed unfractionated mitochondrial extracts by anti-Myc immunoprecipitation to determine the total amount of gRNA associated with WT and non-methylatable RBP16. Fig. 5*A* confirms that both Myc-tagged RBP16 species (WT and TRI) are completely bound by the anti-Myc antibody, while the endogenous RBP16 remains in the non-bound fraction (Fig. 5*A*, compare *lane NB* and *lane B* in *Myc-RBP16-WT* and *TRI*). We then isolated the RNA from each immunoprecipitation fraction and labeled it with α-[^{32}P]GTP using recombinant guanylyltransferase as described above. Fig. 5*B* shows labeled gRNA isolated from anti-Myc immunoprecipitates of total mitochondrial extract from cells expressing either Myc-RBP16-WT or Myc-RBP16-TRI. Two major bands are robustly labeled: a full-length gRNA population of ~60 nucleotides and a truncated population, which are likely to be a previously described population of non-polyuridylated gRNAs (39). Because avid interaction of RBP16 with gRNA requires the poly (U) tail (21), only the full-length population is bound. Quantitation of the full-length gRNA population in each reaction demonstrates that Myc-RBP16-WT bound an average of 44% of the polyuridylated gRNA (Fig. 5*B*, *left panel*), which is in accordance with previously published results (9, 21). In contrast, the non-methylated Myc-RBP16-TRI bound significantly more of the full-length gRNA (an average of 64%; Fig. 5*B*, *right panel*). These data indicate that methylation of RBP16 considerably decreases its association with gRNA.

Methylation of RBP16 Positively Affects Its Association with mRNA—In addition to binding gRNA, RBP16 also associates with several mitochondrial mRNA species.[4] To determine whether methylation of RBP16 leads to a general decrease in RNA binding capacity or whether this effect is specific to gRNA, we quantified mRNAs associated with Myc-RBP16-WT *versus* Myc-RBP16-TRI. To this end, RNA was isolated from anti-Myc immunoprecipitates and analyzed by poisoned
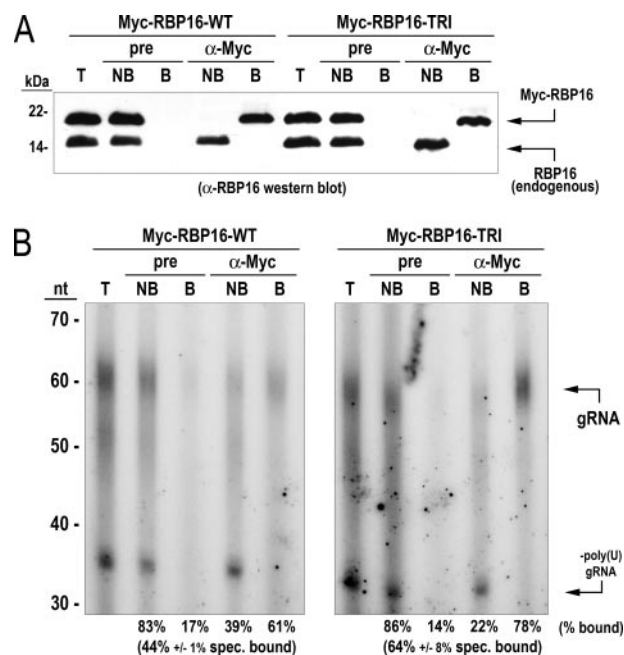

FIGURE 5. **Methylation of RBP16 negatively affects its association with gRNA.** Myc-tagged RBP16 with associated components was isolated from Myc-RBP16-WT and Myc-RBP16-TRI mitochondrial extract by immunoprecipitation with anti-Myc antibodies. *A*, a sample of each fraction was separated by SDS-PAGE on a 17% polyacrylamide gel, transferred to a nitrocellulose membrane, and analyzed by anti-RBP16 Western blot. *B*, the RNA present in each of the fractions shown in *A* was isolated and the gRNA was labeled as in Fig. 2. The *left panel* corresponds to an immunoprecipitation from Myc-RBP16-WT mitochondrial extract, while the *right panel* corresponds to an immunoprecipitation from Myc-RBP16-TRI mitochondrial extract. The *lower band* presumably corresponds to non-uridylated gRNAs that have been described previously (39). *Percent bound* and *percent specifically bound* were calculated as described under "Experimental Procedures." The images used are representative of the results. The percent of gRNA specifically bound to either Myc-RBP16-WT or Myc-RBP16-TRI is shown in *parentheses* below the figures and represents the average and standard deviation from four experiments. *T*, total extract; *NB*, not bound; *B*, bound; *pre*, purified IgG from pre-immune serum; α-*Myc*, purified anti-Myc antibodies.

primer extension for specific transcripts that are known to be affected by RBP16 depletion (CYb, COI, and ND4; Ref. 9). In addition, we also quantified ATPase subunit 6 (A6) mRNA, which is unaffected by RBP16 disruption. We found that 42% of edited and 40% of unedited CYb was specifically bound by Myc-RBP16-WT, as was 35 and 47% of the never-edited ND4 and COI RNAs, respectively (Fig. 6, *left panels*). In contrast, negligible edited A6 mRNA was associated with RBP16 (Fig. 6, *A6E*; unedited A6 RNA was undetectable in this experiment).

To determine whether methylation of RBP16 affects its interaction with mRNA, RNA isolated from an anti-Myc immunoprecipitate of Myc-RBP16-TRI mitochondrial extract was similarly analyzed. Surprisingly, we found that in all cases significantly less mRNA was associated with the non-methylatable Myc-RBP16-TRI as compared with the Myc-RBP16-WT protein (Fig. 6, compare α-*Myc lane B* for *Myc-WT* and *Myc-TRI*). We observed a 53% reduction in COI binding (Fig. 6, *COI*), a 92% reduction in CYbU binding (Fig. 6, *CYbU*), a nearly 100% reduction in CYbE binding (Fig. 6, *CYbE*), and an 86% reduction in ND4 binding (Fig. 6, *ND4*). This is in direct contrast to what was observed for gRNA binding where the association of non-methylatable RBP16 with gRNA was significantly increased compared with WT protein (compare Figs. 6 and 5*B*).
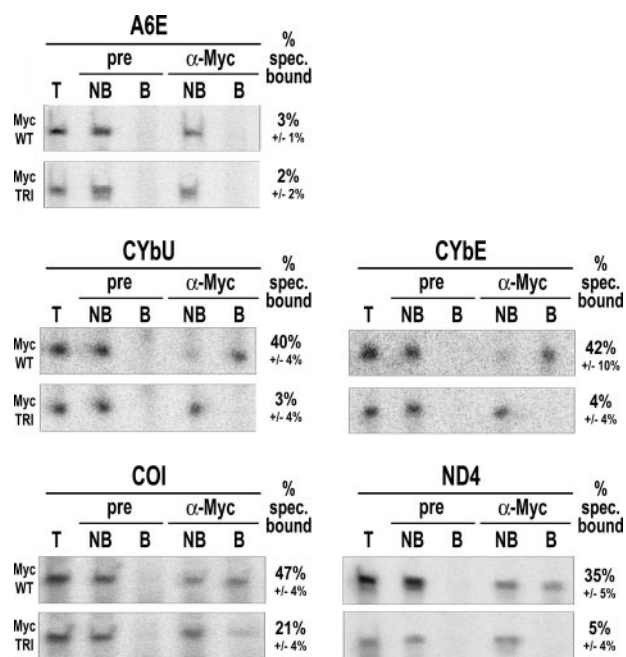
FIGURE 6. **Methylation of RBP16 positively affects its association with mRNA.** The RNA from an anti-Myc immunoprecipitation was analyzed by poisoned primer extension using primers specific for *A6*, *COI*, *CYb*, and *ND4* mRNA. Radiolabeled extension products were separated and analyzed on an 7 M urea, 10% acrylamide gel and visualized by phosphorimager analysis. *A6E*, edited A6; *CYbU*, unedited CYb; *CYbE*, edited CYb. Other labels are as described for Fig. 5. Images are representative of the results. The percent of specifically bound mRNA represents the average and standard deviation from three experiments.
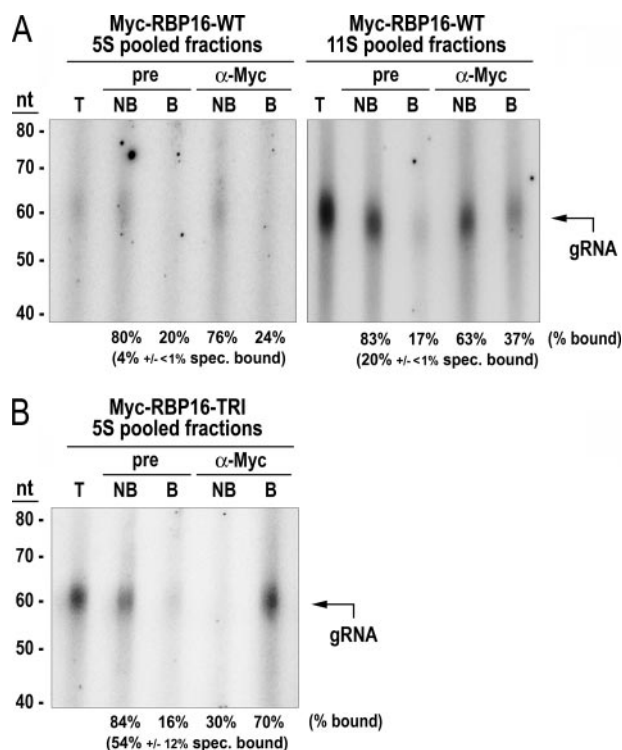

FIGURE 7. **The Myc-RBP16-TRI 5 S complex contains gRNA.** Mitochondrial extract from Myc-RBP16-WT and Myc-RBP16-TRI cells was sedimented, fractionated, and probed with anti-RBP16 antibodies as described in the legend to Fig. 1. Peak Myc-tagged RBP16-containing fractions (3–6 for 5 S WT and 5 S TRI and 11–14 for 11 S WT) were pooled and immunoprecipitated with anti-Myc antibodies to isolate the associated RNA. The RNA present in each of the immunoprecipitation fractions was isolated and labeled for gRNA. Labels are as described for Fig. 5. *A*, the *left panel* corresponds to the immunoprecipitation of the 5 S WT complex, while the *right panel* corresponds to immunoprecipitation of the 11 S WT complex. *B*, immunoprecipitation of the 5 S TRI complex. The images shown are representative of the results. The values under each lane are the average quantification of the percent bound from three experiments. The percent of specifically (*spec.*) bound gRNA in each complex is shown in *parentheses* below the figures and represents the average and standard deviation from three experiments.

Thus, methylation differentially affects the association of RBP16 with various RNA species. These results may indicate a direct effect of methylation on the RNA binding capacity of RBP16 or an indirect effect on protein-protein interactions that influence RBP16 RNA binding specificity and/or affinity.

The Myc-RBP16-TRI 5 S Complex Contains gRNA, while the Myc-RBP16-WT 5 S Complex Does Not—The 5 S complexes formed by Myc-RBP16-WT and Myc-RBP16-TRI appear to differ in their RNA content, since only the complex formed by Myc-RBP16-TRI is RNase sensitive (compare Fig. 3, *A* and *B*). Since total gRNA binding by Myc-RBP16-TRI is increased compared with its wild type counterpart (Fig. 5*B*), these results suggest that the 5 S complex formed by Myc-RBP16-TRI might contain gRNA, while the wild type 5 S complex lacks this component.

To address whether methylation affects RBP16-gRNA interaction in the context of the 5 and 11 S RNP complexes, peak 5 and 11 S Myc-RBP16-WT and 5 S Myc-RBP16-TRI containing fractions (3–6 for 5 S and 11–14 for 11 S) were individually pooled, subjected to anti-Myc immunoprecipitation, and associated RNA isolated. RNA from each immunoprecipitate was labeled with α-[^{32}P]GTP using recombinant guanylyltransferase. Quantitation of the bound gRNA in the Myc-RBP16-WT complexes (Fig. 7*A*) was consistent with the results from the endogenous complexes shown in Fig. 2*B* in that there is significant bound gRNA present in the 11 S complex (Fig. 7*A*, *right panel*) but little or no gRNA in the 5 S complex (Fig. 7*A*, *left panel*).

In contrast to complexes formed by wild type RBP16, when we analyzed the amount of gRNA in the 5 S region of the gradient from the Myc-RBP16-TRI expressing cells, we observed a substantial gRNA population (Fig. 7*B*, *left panel*, lane *T*). Upon anti-Myc co-immunoprecipitation of RNA from the 5 S region of the gradient (Fig. 7*B*, *left panel*), we found that an average of 54% of the gRNA present in this region was specifically bound by the non-methylated RBP16. Thus, a large proportion of the increased gRNA binding by RBP16 observed in total mitochondrial extract (Fig. 5*B*) is reflected in the RNase-sensitive Myc-RBP16-TRI 5 S complex formation. The 11 S region of the gradient from Myc-RBP16-TRI expressing cells did not contain any RBP16-bound gRNA, as expected based on the absence of an 11 S complex formed with the mutant RBP16 (data not shown). Together, the size and RNase sensitivity of the 5 S complex formed by Myc-RBP16-TRI suggest that this complex consists of just RBP16 and associated gRNA in the absence of any other proteins.

To assess the sedimentation properties of a complex containing solely RBP16 and gRNA, we incubated *Escherichia coli* expressed recombinant His-RBP16 with increasing amounts of *in vitro* transcribed gCYb[558] gRNA. These reactions were
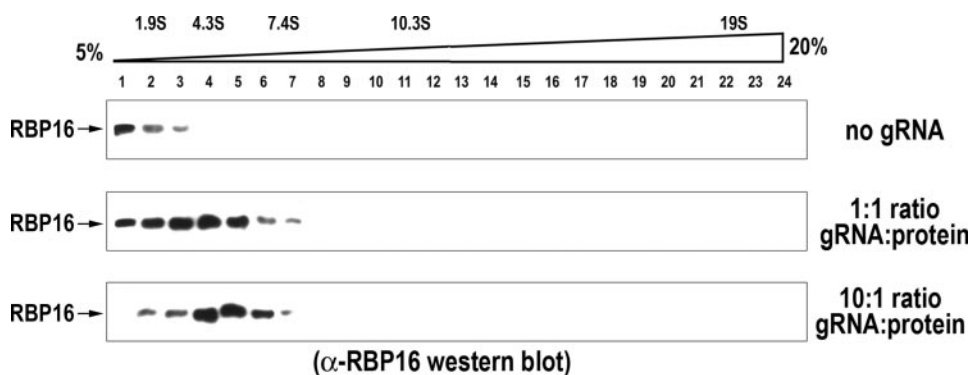
FIGURE 8. **RBP16 and gRNA alone form a 5 S complex.** Recombinant His-RBP16 was incubated in the presence of increasing amounts of *in vitro* transcribed gCYb[558] gRNA prior to gradient sedimentation and fractionation as described in the legend to Fig. 1. Gradient fractions were separated by SDS-PAGE on a 17% gel and probed by anti-RBP16 Western blot. The *upper panel* corresponds to His-RBP16 in the absence of gRNA, the *middle panel* corresponds to His-RBP16 and gCYb[558] at a 1:1 molar ratio, and the *lower panel* corresponds to His-RBP16 in the presence of gCYb[558] at a 1:10 molar ratio.

FIGURE 9. **Model of RBP16 gRNP complex formation.** The model presented is based on the sedimentation of RBP16-containing complexes, along with the evidence of a gRNA component in the WT 11 S and TRI 5 S complexes. For the purposes of this model, endogenous RBP16 and WT-RBP16 can be considered equivalent. *A*, the *upper model* corresponds to an 11 S complex containing a molecule of gRNA bound by methylated RBP16 and associated proteins. The three "*mR*"s represent the three methylated arginine residues. The *lower model* corresponds to a 5 S complex of RBP16 with associated proteins and lacking an RNA component. *B*, this model corresponds to a 5 S complex containing a molecule of gRNA bound by RBP16 alone. The three "*K*"s represent the three arginine residues that have been converted to lysine. This is equivalent to the 5 S complex formed in cells down-regulated for TbPRMT1.

then sedimented on a 5–20% glycerol gradient and fractionated, and RBP16 was detected by Western blot with anti-RBP16 antibodies. While recombinant RBP16 alone sediments at the top of the gradient (Fig. 8, *top panel* (see also Fig. 1*C*)), Fig. 8 (*middle* and *bottom panels*) shows that increasing amounts of His-RBP16 sediment in the 5 S region of the gradient as the amount of gRNA is increased from a 1:1 molar ratio (Fig. 8, *middle panel*) to 10-fold more gRNA (Fig. 8, *bottom panel*). At a 10:1 molar ratio, the majority of His-RBP16 sediments in an ~5 S complex similar to what was observed for Myc-RBP16-

TRI (Fig. 7*B*, *left panel*). From these data we conclude that the only glycerol gradient stable complex formed by non-methylated RBP16 consists of RBP16 bound to gRNA. Together, our data indicate that Arg methylation of RBP16 is required for its association with protein components of the native 5 and 11 S complexes, while the same modification decreases its ability to bind gRNA.

DISCUSSION

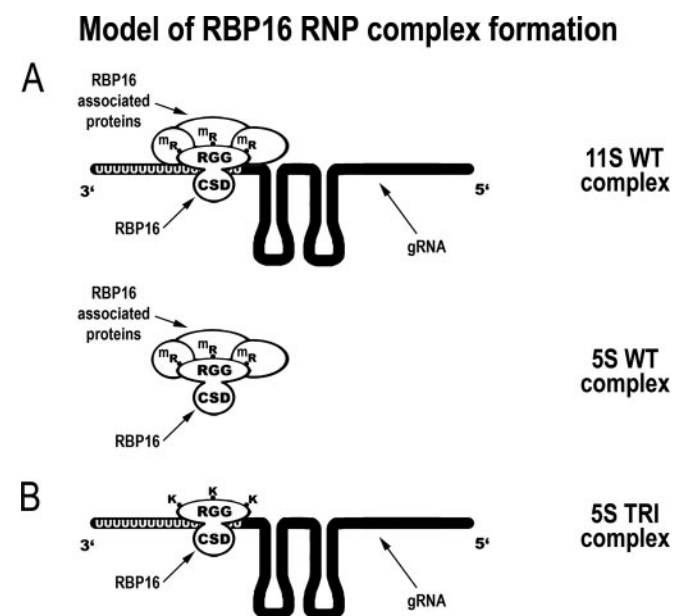In this paper, we identify the major RBP16-containing multiprotein and RNP complexes and determine a role for Arg methylation in their formation. Our data indicate that the majority of endogenous RBP16 is complexed with additional proteins and that a significant fraction of this complex (the 11 S peak) also contains gRNA. Arg methylation, catalyzed by TbPRMT1, is required to maintain the RBP16-protein interactions that mediate 5 and 11 S complex assembly and/or stability. At the same time, this modification decreases the ability of RBP16 to associate with gRNA and increases its association with several mRNAs. Thus, Arg methylation has distinct effects on RBP16 gRNA and mRNA association and gRNP formation.

Our data are consistent with a model of RBP16 gRNP complex assembly that includes RBP16 associating with a core complex of proteins with differential inclusion of gRNA (Fig. 9). The conversion of the 11 S complex into a 5 S complex by RNase treatment (Fig. 1) suggests that the native 5 S complex is a protein subunit of the 11 S complex. The native 5 S complex arguably lacks RNA based on two observations. First, 5 S complexes immunoprecipitated with anti-RBP16 antibodies do not contain gRNA as shown by the absence of guanylyltransferase labeled RNA (Fig. 2*B*). Second, the insensitivity of the 5 S complex to RNase treatment (Fig. 1) further supports the entirely proteinaceous nature of this particle. The interactions involved in native RBP16 complex formation are depicted in the *top panel* of Fig. 9. The 5 and 11 S complexes described above and depicted in Fig. 9*A* were observed with both endogenous RBP16 and exogenously expressed Myc-RBP16-WT (Fig. 3). In contrast, when RBP16 Arg methylation was inhibited either by Arg to Lys mutations or down-regulation of TbPRMT1, both 5 and 11 S complex formation was compromised (Figs. 3 and 4). Under either of these circumstances, the 5 S complexes were converted to monomeric RBP16 upon RNase treatment. The shift to free RBP16 upon RNase treatment demonstrates the presence of RNA in 5 S complexes formed with unmethylated RBP16. Reinforcing this model, immunoprecipitation experiments showed that, unlike native complexes, 5 S complexes formed with unmethylated RBP16 contain substantial amounts of gRNA (Fig. 7*B*, *left*). Finally, *in vitro* RNA binding experiments confirmed that a particle containing solely RBP16 plus gRNA sediments at ~5 S. From these data, we conclude that methylation of RBP16 is required for its association with the

protein components of the native 5 and 11 S complexes but is not required for gRNA association (Fig. 9).

Comparison of total Myc-RBP16-WT and Myc-RBP16-TRI bound gRNA confirmed that methylation is not required for gRNA binding and further showed that methylation actually decreases the capacity of RBP16 to bind gRNA (Fig. 5). Potential mechanisms by which methylation could have a direct negative impact on RBP16-gRNA binding include steric hindrance or disruption of hydrogen bonding. A direct effect of Arg methylation on protein-RNA binding has been reported for the fragile X mental retardation protein, Fmrp (31). Similar to RBP16-gRNA interactions, PRMT1-catalyzed methylation of Fmrp leads to a decrease in binding of this protein to its minimal target RNA sequence. Alternatively, Arg methylation may be required for binding of additional proteins to RBP16, which serve to limit the interaction of RBP16 with gRNA. Such proteins may include components of the complexes identified in this study. The correlated disappearance of the 11 S gRNP and increase in gRNA binding with the Myc-RBP16-TRI protein suggest that the protein components 5 and 11 S complexes serve to decrease, but not eliminate, the RNA binding capacity of RBP16. The effect of associated proteins on the gRNA binding capacity of RBP16 could be manifested by either a decrease in RBP16 RNA binding affinity or a decrease in its specificity for certain gRNA classes. Experiments to dissect these alternatives are currently underway.

Importantly, we also showed that Arg methylation of RBP16 does not cause a global increase in RBP16 RNA binding. Methylation affects RBP16-mRNA interactions in a manner opposite to RBP16-gRNA interactions. Lack of methylation caused a dramatic decrease in RBP16-mRNA association for all mRNAs tested (Fig. 6). The requirement of Arg methylation for optimal RBP16-mRNA association is consistent with our previous genetic studies showing that RBP16 methylation facilitates the mRNA stabilization of the protein functions (11). Together these data suggest that methylation increases the capacity of RBP16 to associate with mRNA, either directly or indirectly, and that increased RBP16-mRNA interaction then facilitates mRNA stabilization.

The 5 and 11 S RBP16-containing complexes identified here constitute the major stable forms of the protein. This suggests that the 5 S complex could represent the primary functional form of RBP16, which interacts with various RNAs, mRNPs, and/or gRNPs to carry out the multiple functions of the protein. Although RBP16 is critical for editing of CYb mRNA (9), it is not currently known whether the 11 S gRNP identified in this study plays a role in facilitating specific RNA editing events or whether it reflects a more global function for RBP16 in gRNA utilization and/or protection. It has been established that RBP16 is associated with 30–40% of total mitochondrial gRNA (*e.g.* Fig. 5*B*) (9, 19, 21). This large percentage of mitochondrial gRNA is almost certainly not required to fulfill the role of RBP16 in CYb mRNA editing (9). Moreover, if the 11 S gRNP complex is required for editing stimulation, we would expect CYb editing to be compromised in TbPRMT1 knock-down cells (11), since TbPRMT1-catalyzed methylation is required for 11 S gRNP formation. Because this is not the case, we instead favor the model in which the 11 S gRNP plays a role in

gRNA packaging. RNA packaging is a common feature of Y-box proteins. For instance, YB-1, in addition to regulating specific transcriptional and post-transcriptional events, is a core component of both translationally active and inactive cytoplasmic mRNPs (46–48). In *Xenopus* oocytes, the Y-box proteins FRGY2 and mRNP3 are major mRNP components that sequester maternal mRNAs from the translational apparatus (49, 50). RBP16 may serve a similar packaging function for gRNAs, with subsequent utilization of different gRNA classes controlled by specific proteins that interact with the major RBP16-containing gRNP. We are currently attempting to identify the protein components of the 5 S and 11 S complexes. Further characterization of these RBP16-containing complexes will provide important insight into the mechanisms by which RBP16 carries out its numerous functions within *T. brucei* mitochondria and how these processes are impacted by protein arginine methylation.

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