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Trypanosoma brucei: functions of RBP16 cold shock and RGG domains in macromolecular interactions

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Abstract

The RNA binding protein RBP16 regulates mitochondrial RNA editing and stability in *Trypanosoma brucei*. To aid in understanding the biochemical mechanisms of RBP16 function, we analyzed the RNA and protein binding capacity of RBP16 and its individual cold shock (CSD) and RGG domains. Both recombinantly expressed domains possess RNA binding activity. However, the specificity and affinity of RBP16 for gRNA is mediated predominantly through the interaction of the CSD with poly(U). The RGG domain contributes to the association between full length RBP16 and gRNA, as it was required for maximal binding. We further demonstrate that both domains contribute to maximal binding of RBP16 to the mitochondrial p22 protein. However, p22 can interact with the CSD alone and stimulate its gRNA binding activity. Thus, the CSD is primary in RBP16 interactions, while the RGG domain enhances the capacity of the CSD to bind both RNA and protein. These results suggest a model for RBP16 molecular interactions.

Keywords: Y-box; Cold shock domain; RGG domain; RNA binding; Trypanosome; RNA editing; RNA stability

1. Introduction

RBP16 is a mitochondrial RNA binding protein from *Trypanosoma brucei* that regulates both RNA editing and stability (Pelletier and Read, 2003). The protein was first identified based on its in vitro gRNA binding ability, and was later shown to be associated with gRNAs in vivo through both immunoprecipitation and in organello crosslinking studies (Hayman and Read, 1999; Militello et al., 2000). The ability of RBP16 to modulate specific RNA editing events is thought to be mediated though its interaction with gRNAs. Detailed RNA binding studies employing gRNA deletion mutants indicated that the oligo(U) tail present at the 3′ end of the gRNA is a major determinant for RBP16 binding (Hayman and Read, 1999; Pelletier et al., 2000). However, high affinity binding of RBP16 apparently requires both sequence-specific interactions with the oligo(U) tail and nonspecific interactions with the encoded portion of the molecule. In addition to binding gRNAs, RBP16 is also associated with 9S and 12S rRNAs and a subset of mRNAs in *T. brucei* mitochondria (Hayman and Read, 1999; unpublished results). The mRNA binding capacity of the protein presumably accounts for its role in regulation of mRNA stability, while rRNA binding suggests additional regulatory functions. RBP16 also directly interacts with a mitochondrial protein termed p22 (Hayman et al., 2001). Association of RBP16 with p22 leads to an increase in the gRNA binding capacity of RBP16 by up to ~10-fold. Thus, the RBP16–p22 interaction is likely to be important in the regulation of trypanosome mitochondrial gene expression.

The mature RBP16 protein consists solely of two domains. At its N-terminus, RBP16 contains a motif known as the cold shock domain (CSD). The CSD is a highly conserved nucleic acid binding motif comprising a 5-stranded β-barrel with RNP1 and RNP2 motifs (Graumann and Marahiel, 1998). Bacterial cold shock proteins (CSPs) consist entirely of one CSD, which generally confers rather weak, nonspecific binding important for the proposed RNA chaperone function of many CSPs, including CspA, B, C, and E (Bae et al., 2000;

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Graumann and Marahiel, 1998; Phadtare and Inouye, 1999). In eukaryotes, the CSD is a key component of the multifunctional Y-box family of proteins, where it mediates specific RNA binding in combination with various auxiliary domains (Matsumoto and Wolffe, 1998). The auxiliary domain of RBP16 comprises its C-terminal region, which is rich in arginine and glycine residues, resembling an RGG RNA-binding motif (Burd and Dreyfuss, 1994). RNA binding by RGG domains is often relatively sequence nonspecific, and one general function of RGG domains is to facilitate binding of associated RNA-binding domains in proteins such as hnRNPA1 and nucleolin (Cobianchi et al., 1988; Yang et al., 1994). However, RGG domains can also be essential for sequence-specific RNA binding as in the hnRNPU protein (Kiledjian and Dreyfuss, 1992). In addition, RGG domains have been shown to mediate protein–protein interactions in several proteins including hnRNPA1 and nucleolin (Bouvet et al., 1998; Cartegni et al., 1996).

To understand the biochemical mechanisms by which RBP16 carries out its functions, it is critical to understand the RNA and protein binding capacities of its component functional domains. For example, if a single domain mediates gRNA binding, then the other putative RNA binding domain of RBP16 may be free to interact with a second type of RNA molecule. Conversely, if both CSD and RGG domains are necessary for gRNA binding, then it will be unlikely that the protein can simultaneously interact with multiple RNA species. If a single domain is required for both RNA and protein binding, this suggests a model whereby the interaction of RBP16 with these molecules takes place consecutively rather than simultaneously. In addition, we previously demonstrated that RBP16 undergoes multiple, mutually exclusive arginine methylation events within its RGG domain (Pelletier et al., 2001). Understanding the specific roles played by the RGG domain in RBP16 molecular interactions will provide insight into the mechanisms by which arginine methylation regulates RBP16 function. In the studies presented here, we characterize the involvement of the CSD and RGG domains of RBP16 in both protein–RNA and protein–protein interactions. Our results indicate that the CSD of RBP16 plays a primary role in mediating both protein–RNA and protein–protein interactions, while the RGG domain stimulates both the RNA and protein binding capacity of the CSD. These findings suggest a model for RBP16 molecular interactions.

2. Materials and methods

2.1. Nucleic acid preparation

A construct encoding the gRNA gA6[14] with a 17 nt 3′ oligo(U) tail was previously described (Read et al., 1994). RNA was synthesized in vitro and internally radiolabeled with [α-^{32}P]UTP (800 Ci/mmol, 10 μCi/μl) (NEN) using the Ambion T7 Maxiscript kit and purified by gel electrophoresis on 6% acrylamide/7 M urea gels.

2.2. Protein expression and purification

A maltose binding protein-RBP16 fusion (MBP-RBP16), Factor Xa-cleaved MBP-p22 and C-terminal His-tagged RBP16 were produced as previously described (Hayman and Read, 1999; Hayman et al., 2001) The CSD and arginine/glycine-rich region (RGG) of RBP16 were produced as fusion proteins containing a C-terminal 6xHis-tag sequence as follows. The CSD and RGG domains of RBP16 were PCR amplified from a Bluescript II SK$^-$ plasmid containing the entire RBP16 open reading frame using the primer pairs (with restriction sites underlined): Tb16K5′exp3 (5′ GCGAATTCCATATGAA CAAGGGTAAGGTGATATCG 3′) and RBP16-3′exphis2 (5′ CCGCTCGAGAGAAGTAACATTCTCGG CAC 3′) for CSD, and RBP16-5′exphis1CP (5′ GC GAATTCCATATGGGAGGAGCCAAACTTCCGTC 3′) and RBP16-3′exphis1 (5′CCGCTCGAGAAAGTC ATCGCTGAAGCTCTG 3′) for RGG. The reaction products were digested and ligated into the *Nde*I/*Xho*I site of pET-21a (Novagen) and transformed into the *Escherichia coli* strain BL21 pLysS (Stratagene). Protein induction and nickel affinity chromatography were as described previously (Hayman et al., 2001). Nickel column-purified proteins were further purified as follows. Full length RBP16 was subjected to Poly(U)-Sepharose chromatography essentially as described (Hayman and Read, 1999), except His-RBP16 was eluted with a step gradient of salt ranging from 200 to 800 mM KCl. CSD was concentrated using the Centricon-3 microconcentrator (Amicon), resuspended in Buffer A as described (Hayman et al., 2001), and loaded onto a Q-Sepharose (Amersham Biosciences) column equilibrated in Buffer A. The column was washed with 10 bed volumes of Buffer A and CSD was eluted with 100 mM KCl in Buffer A while contaminants were retained on the column. RGG was loaded onto a Centricon-10 microconcentrator (Amicon) and centrifuged at 4 °C at 5000*g* until the entire sample passed through the device. RGG was present in the flowthrough while higher molecular weight contaminants were retained. All purified proteins were dialyzed into buffer B (25 mM Hepes, pH 7.5, 30 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM EDTA, and 10% glycerol). The purity and integrity of expressed proteins were examined by Coomassie stains of 15% SDS–PAGE gels. Protein concentrations were determined using the Bio-Rad protein assay with BSA standards.

2.3. UV crosslinking experiments

UV crosslinking of protein to in vitro transcribed RNA was carried out as previously described (Read

et al., 1994). For competition experiments involving ribohomopolymers, 10 fmol of radiolabeled gA6[14] were incubated with RBP16, CSD or RGG (1.6, 1.9, and 11.2 μM, respectively) in the presence of increasing mass excess of unlabeled ribohomopolymer (poly(U), poly(G), poly(A), or poly(C); Sigma) for 20 min at room temperature. Competitor RNAs were added to the reactions prior to the addition of protein. Approximately sixfold higher RGG concentration compared to RBP16 and CSD was used to obtain a comparable UV crosslinking signal and to remain within the linear range of signal detection upon the addition of competitors. Use of different amounts of each protein for competition experiments does not affect the qualitative determination of the relative affinities of the various proteins for homopolymer substrates. For experiments involving p22, proteins (RBP16, 1 μM; CSD, 2 μM; or RGG, 5 μM) were incubated with 10 fmol of radiolabeled gA6[14] and increasing amounts of recombinant p22. Reactions were fractionated by electrophoresis on discontinuous (4–16.5% acrylamide) Tris–tricine gels. UV crosslinking signals were detected by autoradiography, and quantification was performed on nonsaturated autoradiographs using a Bio-Rad model GS-700 imaging densitometer in combination with Molecular Analyst software (version 1.5).

For RNA binding competition experiments presented in Fig. 5, the UV crosslinking assays were performed with 5 fmol of radiolabeled gA6[14] and 1 μM MBP-RBP16. Increasing amounts of 6 × His-tagged RBP16, CSD or RGG (1–20 μM) were included in the reactions. UV crosslinked proteins were resolved by electrophoresis on 15% polyacrylamide Tris–tricine gels. UV crosslinking signals were detected as described above and analyzed with Multi-Analyst version 1.1 (Bio-Rad).

2.4. Gel retardation assays

Reaction conditions were identical to UV crosslinking experiments. Increasing amounts of 6 × His-tagged RBP16, CSD or RGG proteins were incubated with 10 fmol of radiolabeled gA6[14] 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.

2.5. Homopolymer bead assays

Binding of protein to homopolymer beads was essentially as described previously (Vanhamme et al., 1998). Five hundred nanograms of each protein were mixed with 25 μl of agarose beads bound to oligoribonucleotides (poly(U) (Amersham), poly(A) (Amersham), poly(G) (Sigma), or poly(C) (Sigma)) in 0.5 ml of binding buffer (10 mM Tris–HCl, pH 7.4, 2.5 mM $MgCl_2$, 0.5% Triton X-100, with 250 mM or 1 M NaCl). After incubation with gentle mixing for 10 min at 4 °C, beads were pelleted and subjected to three washes, each with 1 ml of binding buffer. SDS–PAGE sample buffer was added to the beads, followed by boiling for 5 min and electrophoresis on 15% SDS–PAGE gels. After electrophoresis, proteins were transferred to nylon membranes (Schleicher and Schuell) and subjected to western blot analysis with rabbit polyclonal α-RBP16 antisera (Hayman and Read, 1999).

2.6. ELISAs

ELISAs were performed in triplicate and carried out in 96-well vinyl plates at room temperature essentially as described (Hayman et al., 2001) with the following modifications. To prepare the immobilized substrate, wells were coated for 3 h with 500 ng of purified protein (6 × His-tagged RBP16, CSD or RGG, or BSA; Sigma) in 50 μl of TBS (50 mM Tris, pH 8.0, 100 mM NaCl). The remainder of the protocol was identical to that published previously (Hayman et al., 2001).

3. Results

3.1. Preparation of RBP16 domain recombinant proteins

Mature RBP16 is a mitochondrial RNA binding protein consisting entirely of two structural domains: an N-terminal cold shock (CSD) and a C-terminal RGG domain (Hayman and Read, 1999) (Fig. 1A). To determine the contribution of each domain to the overall RNA and protein binding characteristics of RBP16, full length RBP16 as well as the individual CSD and RGG domains were expressed as 6 × His-tagged recombinant proteins and purified as described in Section 2. RBP16, CSD, and RGG constituted nearly 100% of the total protein in the purified preparations (Fig. 1B). The predicted molecular weights of the His-tagged constructs are as follows: RBP16, 14.4 kDa; CSD, 8.6 kDa; and RGG, 7 kDa. RBP16 and its RGG domain migrated aberrantly on SDS–PAGE gels compared to their predicted molecular weights presumably due to the basic nature of the RGG domain. The amino acid sequence of RBP16 predicts that each of its domains should be capable of binding RNA. To determine if each protein was in the proper conformation, and to establish the RNA binding activity of the independent domains, we employed UV crosslinking assays. All three proteins bound gRNA (Fig. 2, lanes N), demonstrating that the recombinant proteins are properly folded. These experiments further show that, as predicted from their sequences, both CSD and RGG domains possess the capacity to bind RNA.

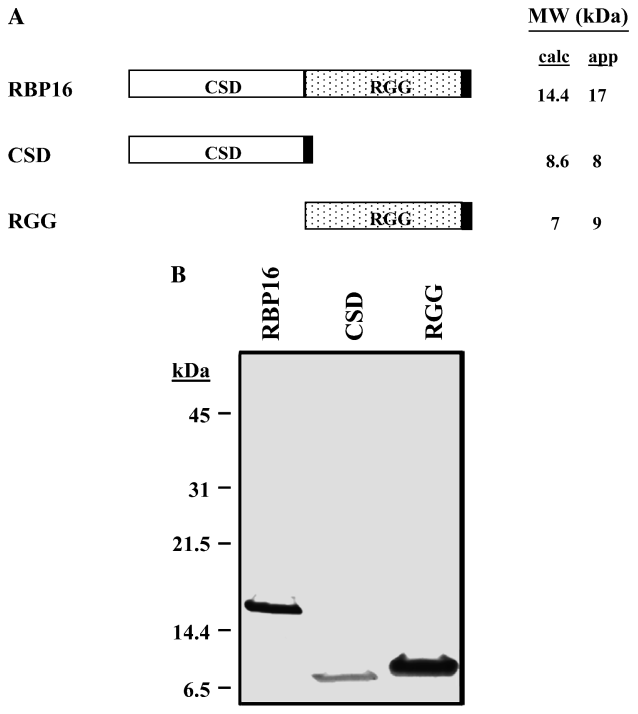

Fig. 1. Domains of RBP16. (A) Schematic representation of the structural domains of RBP16 used in this study. The positions of the CSD and the RGG of RBP16 are indicated. The 6 × His-tag is represented as a black bar. The molecular weights of the proteins as calculated from the amino acid sequences (calc) as well as their apparent molecular masses based on their migration in SDS–PAGE gels (app) are presented to the right. (B) Coomassie-stained SDS–PAGE gel of recombinant full length RBP16 and its constituent CSD and RGG domains (1.25, 0.625, and 2.5 μg, respectively).

3.2. Characterization of RBP16 RNA-binding specificity

Previous experiments employing full length RBP16 and multiple truncated gRNA molecules lead to a model in which we predicted that the CSD domain confers affinity for the gRNA oligo(U) tail, while the RGG domain contributes to the overall affinity of gRNA binding through nonspecific interactions (Pelletier et al., 2000). To directly test this hypothesis, the sequence specificity of CSD and RGG domain RNA binding was examined by UV crosslinking competition assays with various homopolymers (Fig. 2). Full length RBP16 showed comparable affinity for poly(U) and poly(G), whereas no binding to poly(A) or poly(C) was detected. These results are essentially the same as those previously obtained with partially purified native RBP16 and an MBP-RBP16 fusion protein (Hayman and Read, 1999), confirming that the 6 × His-tag does not interfere with the RNA binding properties of RBP16. Competition of CSD–gRNA binding with homopolymers revealed a preference of this domain primarily for poly(U). On the other hand, the RGG domain–gRNA interaction was only effectively competed by poly(G).

To assess the RNA binding specificities of the CSD and RGG domains using an equilibrium binding method, the various protein constructs were used in affinity binding assays with RNA homopolymers immobilized on agarose beads (Vanhamme et al., 1998). RNA–protein interactions were tested at both low (250 mM) and high (1 M) salt concentrations. Bead binding assays confirmed that RBP16 has a preferential affinity for poly(U) and poly(G) (Fig. 3). Binding of RBP16 to homopolymers was moderately salt sensitive, diminishing under high salt binding conditions as previously observed for RBP16–gRNA interactions in electrophoretic mobility shift assays (EMSAs) (Pelletier et al., 2000). As in the UV crosslinking assays, the CSD bound preferentially to poly(U). The homopolymer binding specificity of the RGG domain was even more stringent using this assay, as it exhibited affinity exclusively for poly(G). Interestingly, the CSD–poly(U) interaction was completely insensitive to high salt, presumably reflecting hydrophobic interactions between RNA and phenylalanine residues in the RBP16 RNP1 domain (Pelletier et al., 2000). Conversely, binding of the RGG domain to poly(G) was highly salt sensitive, indicating a large contribution of the electrostatic interactions from this domain. Thus, this experiment reveals the respective contributions of the CSD and RGG domains to the overall salt sensitivity of RBP16–RNA interactions. Taken together, the data obtained by this method confirm the results from the UV crosslinking competition experiments. They demonstrate that the affinity of RBP16 for poly(U) can be attributed to the CSD, while the RGG domain is responsible for the affinity of RBP16 for poly(G). These results are consistent with our previous model in which the CSD interacts with the gRNA oligo(U) tail while the RGG acts to stabilize binding by interaction with the encoded portion of the molecule (Pelletier et al., 2000).

3.3. Characterization of RBP16 RNA-binding affinity

The relative binding of RBP16 and its constituent domains in the homopolymer bead binding assays (Fig. 3) suggested that the full length protein binds RNA with the highest affinity, while the CSD has an intermediate affinity for RNA and the RGG domain binds very poorly. However, we wanted to utilize additional methods to determine the affinity of RBP16 and its domains for gRNA, thereby assessing the relative contributions of the two domains to gRNA binding. To this end, an EMSA was performed in which increasing amounts of RBP16, CSD, and RGG proteins were titrated into a reaction with radiolabeled gA6[14] (Fig. 4). As previously demonstrated, RBP16 formed a complex with gA6[14] beginning at 2.5 μM protein (Hayman and Read, 1999). This complex was replaced with a slower mobility complex beginning at 7.5 μM which increased

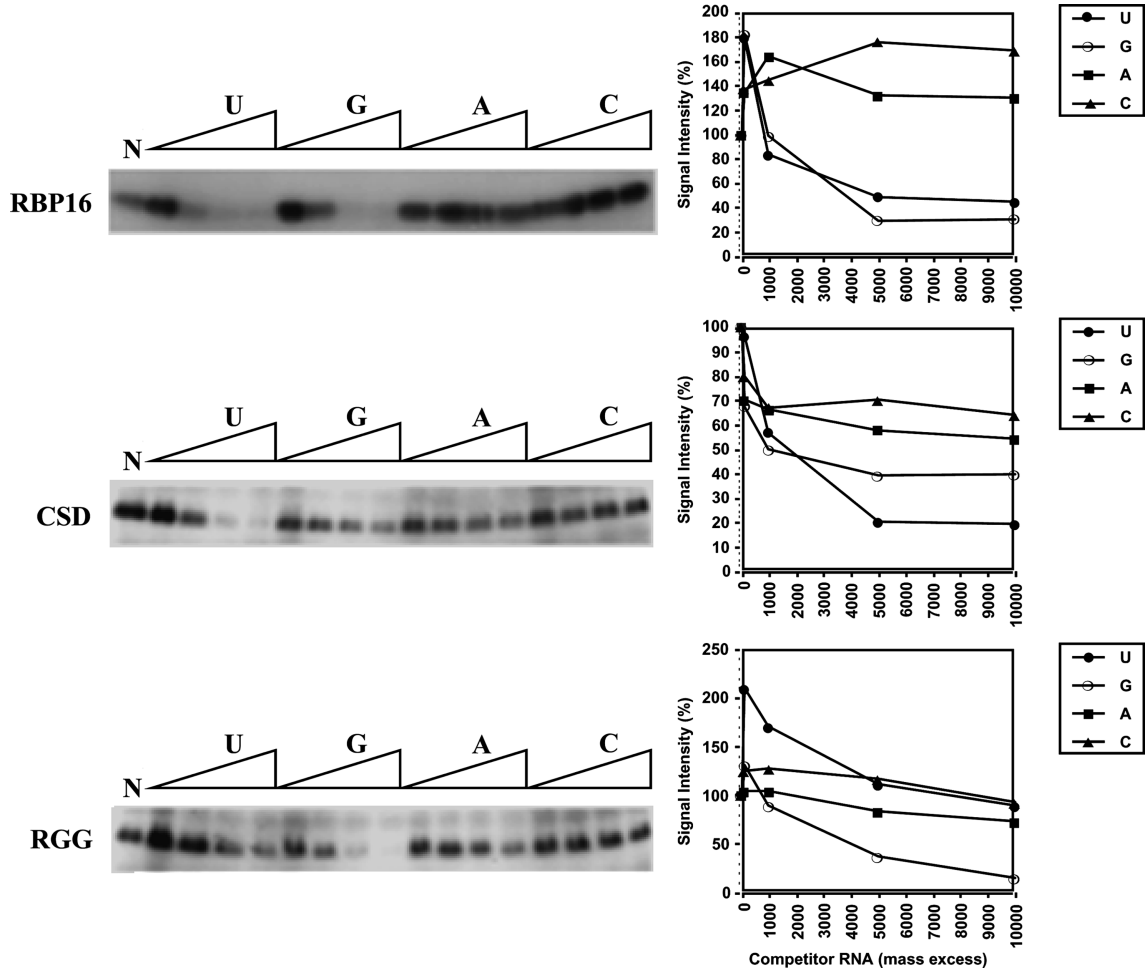

Fig. 2. Characterization of the nucleic acid binding specificity of RBP16 and its domains. Recombinant RBP16, CSD, and RGG proteins were UV crosslinked to radiolabeled gA6[14] in the absence of competitor (N) or in the presence of 100-, 1000-, 5000-, or 10,000-fold mass excess unlabeled ribonucleotide homopolymer competitors. Densitometric analysis of the UV crosslinking signal for each protein construct is shown in the right-hand panel. Competitor levels are plotted on the *x* axis against signal intensity. The UV crosslinking signal in the presence of no competitor is defined as 100%. The increased signal upon addition of polynucleotides in some lanes was not observed in all experiments.

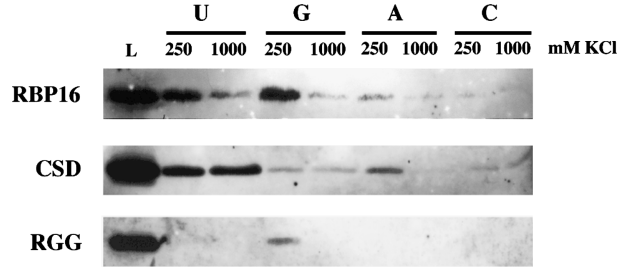

Fig. 3. RNA binding assays using RNA homopolymers immobilized on agarose beads. Recombinant RBP16, CSD or RGG proteins were incubated with the indicated oligonucleotides bound to agarose beads in the presence of 250 mM or 1 M NaCl. After several washes, 50% of the bound proteins were analyzed by Western blot using α-RBP16 antibodies. Lane L is equivalent to 10% of the total amount of protein used in the binding reactions.

in size with increasing protein concentration (Fig. 4). The appearance of two shifted forms was initially postulated to represent two RBP16 molecules bound to one gRNA molecule. However, an EMSA experiment titrating RBP16 in the range between 2 and 7.5 μM revealed a gradual shift from a faster mobility complex to a slower mobility complex (data not shown). Thus, the shift in mobility with increasing protein concentration appears to represent a conformational change, rather than one versus two molecules of protein bound to one RNA molecule.

The CSD also exhibited gRNA binding ability by EMSA, although binding of the CSD to gRNA followed a different pattern than that observed with full length RBP16. The CSD–gRNA interaction produced a mobility shift appearing as a doublet, which increased in intensity at increasing protein concentration. Little change in complex mobility was observed, indicating that the characteristic alteration in RBP16 protein–RNA complex migration upon protein titration may be attributed to the RGG domain. Moreover, full length RBP16 and the CSD differed dramatically in regard to

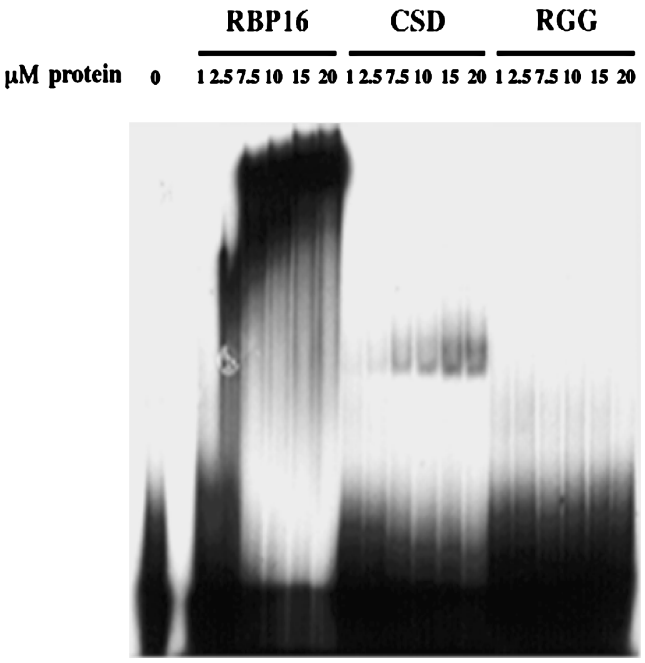

Fig. 4. EMSA analysis of the gRNA binding affinity of RBP16, CSD, and RGG proteins. Increasing concentrations of recombinant RBP16, CSD, and RGG proteins were incubated with 10 fmol of radiolabeled gA6[14]. Following electrophoresis on an 8% nondenaturing acrylamide gel, RNA–protein complexes were detected by autoradiography.

the amount of shifted RNA obtained at a given protein concentration. RBP16 bound a significantly higher proportion of the total RNA than the CSD, indicating that the full length protein has a higher affinity for gRNA than does the CSD domain alone. These results are consistent with those obtained in the homopolymer bead assays. While complexes were observed with both RBP16 and CSD at the range of protein concentrations tested, the RGG domain failed to produce a mobility shift at any concentration tested. This indicates that the affinity of the RGG–RNA interaction is too low to withstand EMSA conditions and suggests that the CSD provides a greater contribution to the RBP16–gRNA interaction than does the RGG domain.

To further examine the relative affinities of RBP16 and its domains for gRNA, RNA binding competition experiments (Lisitsky et al., 1994) were performed in which a constant amount of MBP-RBP16 was UV crosslinked to radiolabeled gA6[14] in the presence of increasing fold excess of 6 × His-tagged RBP16, CSD or RGG proteins (Fig. 5). The ability of the His-tagged full length or domain proteins to compete the binding of MBP-RBP16 to gA6[14] was compared to obtain the relative affinities of each protein for gRNA. An I_{50} value was defined as the concentration of competitor protein that inhibited the binding of MBP-RBP16 to gA6[14] by 50% in our experimental system (Lisitsky et al., 1994). Consistent with the results from EMSAs, RBP16 competed binding of MBP-RBP16 most efficiently, with an

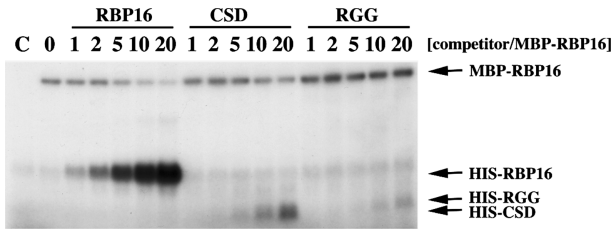

Fig. 5. Competition of RBP16 domain constructs for binding of gA6[14] to MBP-RBP16. RBP16 fused to maltose binding protein (MBP-RBP16, 1 μM) was incubated with increasing molar excess (1-, 2-, 5-, 10-, or 20-fold) of the competitor protein (6 × His-tagged RBP16, CSD or RGG), followed by addition of radiolabeled gA6[14] (5 fmol) and UV crosslinking. Protein–RNA complexes were resolved by electrophoresis and detected by autoradiography. C, no added protein, 0, no added competitor.

I_{50} value between 5 and 10 μM. The CSD competed less well, with 50% competition at greater than 20 μM. RGG was not able to compete MBP-RBP16 binding to gA6[14] at any of the concentrations tested, although it was itself able to crosslink to gA6[14] at high concentrations. The ability of the RGG domain to interact with gRNA under UV crosslinking conditions (Figs. 2 and 5) but not in EMSAs (Fig. 4) indicates that the RGG domain has low affinity for gRNA in the absence of stabilization from either the CSD or UV crosslinking. Although the RGG domain alone cannot stably bind gRNA, its presence in RBP16 is nevertheless indispensable, since it significantly enhances the gRNA binding capacity of the CSD in the context of the full length protein (compare RBP16 lanes to CSD lanes in Fig. 4). Overall, these experiments indicate that while the CSD contributes significantly more to the affinity of RBP16 for gRNA than does the RGG domain, maximal RBP16–gRNA interactions require both domains.

3.4. Characterization of RBP16 protein binding specificity

We previously identified by affinity chromatography an RBP16-associated polypeptide from mitochondrial lysates, which we termed p22 (Hayman et al., 2001). Since p22 is acidic (p*I* 4.5), while the RGG domain of RBP16 is highly basic (p*I* 11), we speculated that p22 would interact with RBP16 through the RGG domain via charge interactions, similar to the interaction of human and murine p22 homologs with the arginine-rich basic domain of HIV Rev (Luo et al., 1994; Tange et al., 1996). To determine the relative contributions of the CSD and RGG domains to the RBP16–p22 interaction, we examined binding of recombinant p22 to RBP16 and its domains by ELISA (Hayman et al., 2001). As shown in Fig. 6, the CSD was the only domain with appreciable binding to p22. Interaction of p22 with the CSD was specific and increased as a function of p22 concentration. Contrary to our expectations, RGG did not demonstrate a measurable interaction with p22 above

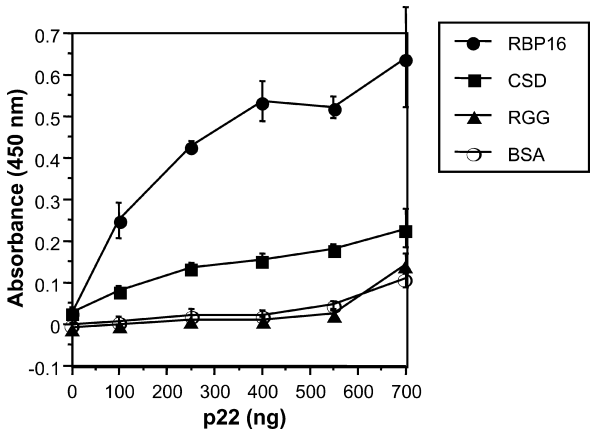

Fig. 6. p22 interacts with the CSD of RBP16. Wells of a 96-well plate were coated with 6 × His-tagged RBP16, CSD or RGG proteins or BSA and challenged with increasing amounts of recombinant p22. Interactions were detected with antibody against p22 and horseradish peroxidase-conjugated secondary antibody. The wells were incubated with a chromogenic substrate, and absorbance was measured at 450 nm. Reactions were performed in triplicate, and the mean and standard deviation are shown.

control levels. This experiment demonstrates that p22 interacts with RBP16 through the CSD. However, because the level of binding of p22 to the CSD was 3–4 times lower than to full length RBP16, both domains are required for the most favorable interaction. These results lend support to the physiological relevance of our previous finding that p22 specifically modulates the RNA binding affinity of RBP16, since the increased affinity is not a result of nonspecific charge–charge interactions.

3.5. Stimulation of CSD gRNA binding activity by p22

We next wanted to confirm that the CSD domain alone is capable of binding p22 and to analyze whether p22 can modulate the gRNA binding affinity of the CSD as it does for full length RBP16 (Hayman et al., 2001). To address these questions, p22 was titrated into a UV crosslinking reaction with either recombinant RBP16, CSD or RGG protein and radiolabeled gA6[14]. As previously observed (Hayman et al., 2001), p22 significantly stimulated the gRNA binding ability of RBP16, up to about sixfold in this experiment (Fig. 7). p22 effected a smaller, but reproducible increase in the gRNA binding capacity of the CSD, up to approximately twofold. These results confirm the ELISA data presented in Fig. 6, showing that p22 interacts with the CSD domain of RBP16. Binding of the RGG domain to gRNA was not affected by the presence of p22. Thus, the interaction between p22 and the RBP16 CSD contributes significantly to the modulation of RBP16's gRNA binding ability.

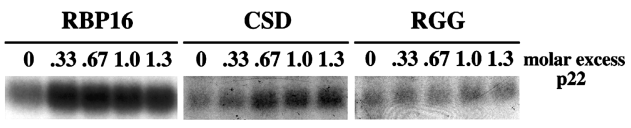

Fig. 7. p22 stimulates CSD–gRNA binding. Recombinant RBP16, CSD or RGG proteins were UV crosslinked to in vitro transcribed gA6[14] either in the absence or presence of increasing molar excess of p22. Samples were analyzed by electrophoresis and autoradiography. The p22 molar excesses were calculated based on a p22 trimer (Hayman et al., 2001).

4. Discussion

RBP16 binds multiple classes of mitochondrial RNAs (Hayman and Read, 1999; Pelletier et al., 2000) and plays a role in regulating both RNA editing and RNA stability in *T. brucei* mitochondria (Pelletier and Read, 2003). A comprehensive understanding of the biochemical function of RBP16 requires the characterization of the RNA and protein binding potentials of its constituent functional domains. In this report, we describe the analysis of the role of the CSD and RGG domains of RBP16 in both protein–RNA and protein–protein interactions. We find that the CSD is of primary importance in both protein–protein and protein–RNA binding, while the RGG domain is required to promote maximal interaction of full length RBP16 with both protein and RNA.

Both the CSD and RGG domains of RBP16 were demonstrated to possess RNA binding activity. However, both domains displayed RNA binding affinities and specificities that differed significantly from each other and from full length RBP16. Based on the data presented here, we can expand our previous model of the RBP16–gRNA interaction, which suggested that binding of RBP16 to gRNA involves both sequence-specific and nonspecific protein–RNA contacts (Pelletier et al., 2000). The specificity of the CSD for poly(U) indicates that the CSD mediates sequence-specific binding of RBP16 to gRNA through the oligo(U) tail. The RGG domain, which displays preferential binding to poly(G)-containing homopolymers, then presumably stabilizes the RBP16–gRNA interaction through nonspecific contacts with the encoded region of the gRNA molecule, increasing the overall affinity of the RBP16–gRNA interaction. EMSA studies indicate that the CSD provides a greater contribution to RBP16–gRNA binding than does the RGG domain, since the CSD alone is able to form stable complexes with gRNA under these conditions while the RGG domain is not. Thus, it follows that the specificity of RBP16 for gRNA is mediated predominantly through the affinity of the CSD for poly(U). The preference of the RGG domain for sequences containing G ribonucleotides also allows for the possibility that the RGG domain could facilitate interaction with G-rich pre-mRNAs, aiding in the assembly of a ribonucleoprotein complex containing pre-mRNA as well as gRNA. However, as is the case with

gRNA, the RGG domain alone is similarly unable to stably bind mRNA (data not shown). This renders it unlikely that a single RBP16 molecule can bind simultaneously gRNA and mRNA through its CSD and RGG domains, respectively. We have previously reported that mRNA competes RBP16–gRNA interactions as efficiently as does gRNA (Pelletier, et al., 2000), contrary to what would be expected if both RNA molecules were simultaneously bound. Moreover, attempts to demonstrate formation of a ternary complex containing RBP16, gRNA, and mRNA have proven unsuccessful (unpublished results). Taken together, our results strongly suggest that RBP16 binds RNA with a 1:1 stoichiometry.

Despite the low RNA binding affinity of the RGG domain, this domain does not function merely in an accessory manner in the RBP16–gRNA interaction. Its presence is indispensable to the RNA binding activity of RBP16. In support of this conclusion, not only is the gRNA binding affinity of RBP16 significantly diminished in the absence of the RGG domain in both EMSAs and UV crosslinking experiments, the pattern of protein–RNA complex formation in EMSAs is altered (Fig. 4). Inclusion of the RGG domain in the full length protein is expected to add a higher degree of flexibility to the protein. C-terminal auxiliary domains in proteins such as nucleolin and FRGY2 are reported to adopt an extended PPII helix-like conformation, with an absence of α-helical or β-strand regions (Ghisolfi et al., 1992; Manival et al., 2001). Likewise, the RGG domain of RBP16 lacks α-helical or β-strand regions and is also believed to adopt an extended structure (Manival et al., 2001). In addition to contributing to protein flexibility, the RGG domain may also play a role in facilitating multiple RNA conformations in a manner similar to that reported for the FRGY2 and nucleolin C-terminal domains, which modulate the RNA conformation induced by their CSD and RRM domains, respectively (Manival et al., 2001). The predicted ability of the RGG domain of RBP16 to promote both increased protein flexibility and multiple RNA conformations likely contributes to the observation of multiple RBP16–gRNA complexes as compared to the single doublet obtained in the absence of the RGG domain.

Recently, our laboratory confirmed a direct interaction between the RBP16 and p22 proteins (Hayman et al., 2001). We previously reported that p22 promotes the association of RBP16 with RNA, dramatically stimulating the RNA binding properties of RBP16 (Hayman et al., 2001). In this report, we show that both CSD and RGG domains contain determinants for p22 binding. Moreover, p22 interacts with the CSD of RBP16 and enhances the RNA binding activity of that domain approximately twofold. These results suggest that the CSD of RBP16 is the domain primarily responsible for mediating the RBP16–p22 interaction, as well as the observed modulation of the RNA binding activity of RBP16 by p22. Nevertheless, the presence of the RGG domain is important for both the level of protein–protein interaction as well as the degree of stimulation of RNA binding activity. This is evidenced by the significantly lessened ability of p22 to interact with the CSD and to stimulate its RNA binding capacity compared to full length RBP16, by approximately three to fourfold in each case. It is not clear whether the lower level of stimulation of gRNA binding activity of the CSD is due to the compromised protein–protein interaction, or the reduced ability of the CSD to interact with gRNA in the absence of the RGG domain. However, the similar level of abatement of protein–protein interaction and RNA binding enhancement (three to fourfold) suggest that the diminished effect of p22 on CSD–gRNA binding is mainly a result of a decreased degree of protein–protein interaction. The relative roles of the CSD and RGG domains in RBP16 protein–protein interactions is reminiscent of what has been reported for protein–protein interactions of another Y-box protein, YB-1. Partnering of YB-1 with the SRp30c splicing factor involves both a high affinity binding site in the N terminal CSD-containing domain of YB-1 as well as lower affinity contacts within the C terminal region of the protein (Raffetseder et al., 2003).

Our finding that the CSD domain of RBP16 is a primary determinant in both p22 and RNA binding suggests that steric constraints may hinder formation of a stable trimeric p22–RBP16–gRNA complex. Instead, p22 may act catalytically to stimulate the RNA binding ability of RBP16, interacting only transiently with either free RBP16 or gRNA-bound RBP16. We are exploring further the mechanism by which p22 stimulates the RNA binding activity of RBP16 and the in vivo implications of the RBP16–p22 interaction.

Finally, RBP16 undergoes the mono-, di-, and trimethylation of several arginine residues in the RGG domain (Pelletier et al., 2001). Since some of these methylation events are mutually exclusive, methylation of key arginine residues within the RGG domain of RBP16 may play a role in regulating its function. We show in this study that the RGG domain, while not sufficient, is necessary in facilitating the interaction of RBP16 with both RNA and protein. Importantly, these results suggest that modification of the RGG domain by methylation has the potential to modulate both protein–protein and protein–RNA associations of full length RBP16. We are currently investigating the impact of methylation of arginine residues in the RGG domain on RBP16 macromolecular interactions and function.

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