Disulfide Bond Formation between RNA Binding Domains Is Used to Regulate mRNA Binding Activity of the Chloroplast Poly(A)-binding Protein*

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Binding of the chloroplast poly(A)-binding protein, RB47, to the psbA mRNA is regulated in response to light and is required for translation of this mRNA in chloroplasts. The RNA binding activity of RB47 can be modulated in vitro by oxidation and reduction. Site-directed mutations to individual cysteine residues in each of the four RNA binding domains of RB47 showed that changing single cysteines to serines in domains 2 or 3 reduced, but did not eliminate, the ability of RB47 to be redox-regulated. Simultaneously changing cysteines to serines in both domains 2 and 3 resulted in the production of RB47 protein that was insensitive to redox regulation but retained the ability to bind the psbA mRNA at high affinity. The poly(A)-binding protein from Saccharomyces cerevisiae lacks cysteine residues in RNA binding domains 2 and 3, and this poly(A)-binding protein lacks the ability to be regulated by oxidation or reduction. These data show that disulfide bond formation between RNA binding domains in a poly(A)-binding protein can be used to regulate the ability of this protein to bind mRNA and suggest that redox regulation of RNA binding activity may be used to regulate translation in organisms whose poly(A)-binding proteins contain these critical cysteine residues.

Translational regulation is the predominant mechanism for controlling gene expression in the chloroplast of plants and algae (1–3). In Chlamydomonas reinhardtii, translational regulation of the chloroplast psbA gene, which encodes the D1 protein (a major component of photosystem II), appears to require the binding of a complex composed of four nuclear-encoded proteins (4). The 47-kDa member of this protein complex (RB47) has been identified as a polyadenylate-binding protein (PABP)†(5). Physiological studies have shown that both RB47 RNA binding activity and psbA translation are activated in a light-dependent manner (4). Characterization of nuclear mutants lacking RB47 has shown that this protein is required for binding of the complex to the 5′-untranslated region (UTR) of the psbA mRNA (6). Absence of RB47 results in the failure of the psbA mRNA to associate with polyribosomes, and hence the loss of translation, suggesting that RB47 acts as a message-specific translation initiation factor for the psbA mRNA (6). Binding of the RB47 protein to the 5′-UTR of the psbA mRNA can be modulated in vitro by oxidation and reduction reactions (7).

The translational machinery of the chloroplast has similarities to prokaryotes including 70 S ribosomes and mRNAs that lack poly(A) tails and 7-methyl-G caps. PABPs have not as yet been identified in prokaryotic organisms. However, the 5′-UTR of the psbA mRNA is A/U-rich and contains two stretches of A residues that have been identified as the primary binding site for the RB47 proteins (8). RB47 contains the four RNA recognition motifs (RRMs), which are highly conserved in all PABPs. The structure of RRM1 and RRM2 of the human PABP has been determined (9) and shows a ββαββαβ secondary structure for each of the RNA binding domains (9–11). The four stranded β-sheet and two antiparallel α-helices form a structure that is characteristic of the ribonucleoprotein motif (12). In vitro RNA binding studies have revealed that all four RRMs are required for high affinity and high specificity RNA binding, but individual RRMs are capable of binding RNA in a nonspecific manner (13, 14).

Sequence alignment with other PABPs shows RB47 to be unique in containing at least one cysteine residue within each of the four RRMs. To identify the role, if any, of the cysteine residues of RB47 in redox-regulated binding activity, each of the cysteines was mutated to serine using site-directed mutagenesis. We show that redox regulation of psbA mRNA binding activity is slightly reduced in single-site mutations to the cysteine residues of the second and third RRMs, but not in the other two RRM domains. A double mutant in which cysteines are replaced by serines in both the second and third RRMs results in a protein that is insensitive to oxidizing or reducing conditions but retains the ability to bind the psbA mRNA at high affinity. PABP from yeast does not contain cysteines in RRM2 or RRM3, and we show that, unlike RB47, the yeast poly(A)-RNA binding is not regulated by redox status. These data suggest that disulfide bond formation between the second and third RRMs of RB47 is responsible for the redox regulation of psbA mRNA binding activity. The formation of disulfide bonds between the second and third domains may generate a tertiary structure that precludes RNA access. These data thus distinguish PABPs containing cysteine residues in the second or third RRM domains, such as RB47, from PABPs that lack these residues, as being capable of redox regulation of mRNA binding and translation.

**EXPERIMENTAL PROCEDURES**

Site-directed Mutagenesis of RB47—The C55S, C143S, C259S, C336S, and C353S RB47 plasmids were generated using a Quickchange site-directed mutagenesis kit (Stratagene). In all five cases, the individual cysteine residues were changed to serines according to the manufacturer’s instructions.

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† The abbreviations used are: PABP, polyadenylate-binding protein; UTR, untranslated region; RRM, RNA recognition motif; PAGE, polyacrylamide gel electrophoresis; Ni-NTA, nickel-nitrilotriacetic agarose; GMS, gel mobility shift; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); wt, wild type; DTT, dithiothreitol.
ufacturer's instructions with slight modifications. Primers consisting of 24 bases were designed to anneal to the same sequence on opposite strands of the plasmid. These primers were purified using a denaturing acrylamide gel and visualized by ultraviolet shadowing. Gel extraction of the primers was accomplished by overnight soaking in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0). In each mutagenesis, the primers were used at a concentration twice that of the plasmid. Following polymerase chain reaction amplification of the RB47 cDNA cloned into a pET19b plasmid (Novagen), the DNA samples were extracted with phenol and chloroform, precipitated and washed with ethanol, and resuspended in TE buffer. The amplified plasmids were then transformed into E. coli cells (Top 10, Invitrogen). Colonies were picked, and plasmid purification was done by ion exchange using the resin referred to as RRMs.

Sequencing of RB47 Mutants—Plasmids obtained from the Quick-Change site-directed mutagenesis kit were verified by sequencing using 7-deaza-dGTP and the T7 Sequenase kit (Amersham). In all cases, the change was verified by sequencing using the primers was accomplished by overnight soaking in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0). In each mutagenesis, the primers were used at a concentration twice that of the plasmid. Following polymerase chain reaction amplification of the RB47 cDNA cloned into a pET19b plasmid (Novagen), the DNA samples were extracted with phenol and chloroform, precipitated and washed with ethanol, and resuspended in TE buffer. The amplified plasmids were then transformed into E. coli cells (Top 10, Invitrogen). Colonies were picked, and plasmid purification was done by ion exchange using the resin referred to as RRMs. All PABPs contain a cysteine residue in the second or third RRM domains, may be able to contain a cysteine residue in the 2 sheet of RRM1, and all except C. reinhardtii contain a cysteine residue in the 3 sheet of RRM4. As shown in Fig. 1, members of the PABP family show high sequence conservation, and all contain the four subdomains referred to as RRMs. All PABPs contain a cysteine residue in the 2 sheet of RRM1, and all except C. reinhardtii contain a cysteine residue in the 3 sheet of RRM4. RB47 is unique even among plants in containing a cysteine residue in the a4 helix of RRM3. Cysteine residues in the 2 sheet of RRM4 appear to be a characteristic of plant PABPs, whereas cysteine residues in the 2 sheet of RRM4 are dominant in animal PABPs. RB47 contains cysteine residues in both the 2 sheet and 3 sheet of RRM4.

Poly(A)-binding Protein from S. cerevisiae Is Incapable of Regulation by Redox Status—Both RB47 and S. cerevisiae PABP were expressed in E. coli cells with His10 amino-terminal extensions and isolated by Ni-NTA resin chromatography. The purified proteins were subjected to oxidation with 3 mM DTNB followed by dialysis to remove the oxidant. A gel mobility shift (GMS) assay with labeled poly(A)-RNA showed that the PABP from S. cerevisiae (Fig. 2, lanes 4–6), which does not have cysteines in the second and third RRMs, does not show redox-regulated binding, whereas the wt RB47 shows the expected redox regulation (Fig. 2, lanes 1–3). These data suggest that RB47 and other poly(A)-binding proteins, which contain cysteine residues in the second or third RRM domains, may be able to reduce the binding activity through redox regulation.

RESULTS

Amino Acid Sequence Alignment of Poly(A)-binding Proteins—As shown in Fig. 1, members of the PABP family show high sequence conservation, and all contain the four subdomains referred to as RRMs. All PABPs contain a cysteine residue in the 2 sheet of RRM1, and all except C. reinhardtii contain a cysteine residue in the 3 sheet of RRM4. RB47 is unique even among plants in containing a cysteine residue in the a4 helix of RRM3. Cysteine residues in the 2 sheet of RRM4 appear to be a characteristic of plant PABPs, whereas cysteine residues in the 2 sheet of RRM4 are dominant in animal PABPs. RB47 contains cysteine residues in both the 2 sheet and 3 sheet of RRM4.

Oxidation and Reduction of Purified Proteins—Purified proteins were oxidized by incubation with 3 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) at room temperature for 10 min. To remove the oxidant, proteins were dialyzed against 5000 volumes of buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM KOAc, 0.2 mM EDTA, and 20% glycerol for 4 h at 4 °C. A sample of each oxidized protein was rerun by incubation at room temperature for 10 min with 25 mM dithiothreitol (DTT).

Gel Mobility Shift (GMS) Assays—P-Labeled, T7 transcribed RNA corresponding to the 5′-UTR of the psbA mRNA was denatured at 80 °C for 2 min and then reannealed at 37 °C for 10 min. 5 μl of dialysis buffer (20 mM Tris-Cl, pH 7.5, 100 mM KOAc, 0.2 mM EDTA, and 20% glycerol) was added to 0.5 μl of total mRNA from a C. reinhardtii mutant lacking psbA mRNA (specific competitor). Reactions were incubated at room temperature for 10 min and then separated on a nondenaturing 1 × TBE, 5% polyacrylamide gel. The gel was exposed to autoradiography to detect protein-RNA complexes.

Redox Regulation of mRNA Binding Activity

Fig. 1. Amino acid sequence alignment of individual RRMs from RB47 and other poly(A)-binding proteins, which contain cys- teine residues in the second or third RRM domains, may be able to reduce the binding activity through redox regulation.

Figure 1: Amino acid sequence alignment of individual RRMs from RB47 and other poly(A)-binding proteins.
to activate RNA binding activity in a redox-dependent manner, whereas those that lack these residues are insensitive to redox regulation.

**Single Mutations of Cysteine to Serine Residues Produce an RB47 Protein That Retains Sensitivity to Redox Status**—RB47 and single-site mutants to each of the cysteines within the RB47 protein were expressed in *E. coli* and purified by Ni-NTA resin chromatography. Purified proteins were subjected to oxidation and reduction as described above. A GMS assay was used to determine the effect of mutations at each cysteine residue on the ability of RB47 to bind mRNA under oxidizing and reducing conditions. As shown in Fig. 3A, single-site mutations to each of the cysteine residues of RB47 resulted in the production of proteins that were still capable of being regulated by oxidation and reduction. Mutations into the second (C143S, lane 4) and third (C259S, lane 5) cysteines resulted in the production of RB47, which showed an incomplete loss of *psbA* mRNA binding activity under oxidizing conditions.

To show that oxidation did not result in a nonspecific inactivation of the RB47 protein or that a mutation to cysteine residues did not simply abolish the RNA binding capacity of the protein, 25 mM DTT was used to rereduce the oxidized proteins. As shown by GMS assay in Fig. 3B, psbA mRNA binding is reacquired in each of the proteins by treatment with the reducing agent. These data show that the affinity of RB47 for the *psbA* message is linked to the redox status of the RB47 protein and that elimination of any single cysteine residue does not dramatically affect the ability of RB47 to bind RNA.

**Simultaneous Mutations in the Second and Third RRMs of RB47 Block Inactivation of psbA mRNA Binding by Oxidation**—A double mutant was created by excising a fragment from the C259S mutant containing the mutation in the third RRM and ligating this fragment as a replacement into the C143S mutant construct. This C143S/C259S double mutant was sequenced to confirm that the plasmid contained both mutations changing serines to cysteines in the second and third RRM domains. The C143S/C259S protein was expressed in *E. coli* and isolated on a Ni-NTA resin column. A GMS assay was
preformed with untreated, oxidized, and rereduced double mutant versus its wt RB47 counterpart. As shown in Fig. 4, the C143S/C259S protein (lane 2) expressed in E. coli binds psbA mRNA with only a slight decrease in affinity as compared with the wt RB47 protein (lane 1). Oxidation of the wt RB47 protein resulted in the complete loss of RNA binding, whereas oxidation of the C143S/C259S protein resulted in only a slight reduction in binding activity (compare lane 3 with 4). Rereduction of the double mutant protein had little effect on binding activity, while the wt RB47 showed the expected reactivation by DTT treatment (lanes 5 and 6). These data suggest that disulfide bond formation between the second and third cysteine is primarily responsible for the inactivation of RNA binding activity of RB47 by oxidation.

**DISCUSSION**

RB47 plays a major role in the translation of the chloroplast psbA mRNA. Translation of this mRNA requires RB47 binding to the 5'-UTR of the mRNA, and RB47 binding activity and psbA translation are both regulated in a light-dependent manner. Exposure of *C. reinhardtii* cells to light initiates a chain of redox events that leads to the reduction of RB47, thereby resulting in the activation of RNA binding activity and mRNA translation. RB60, a chloroplast-localized protein-disulfide isomerase, is capable of regulating the binding activity of RB47 by reversibly changing the redox status of the RB47 protein (15). Cysteine residues found within the four RRMs of RB47 are the logical targets of this RB60-catalyzed disulfide bond formation. Alignment of PABPs from a number of species showed that, as a family, cysteine residues are conserved within RRM1 only, although several PABPs have cysteines in each of the four RRMs.

Single-site mutations in which cysteine residues were converted to serines within each of the four RRM domains of RB47 showed that none of these residues had an effect on the ability of the RB47 protein to bind RNA. Alteration of any single cysteine also failed to produce a protein that completely lacked redox regulation. However, mutations to the cysteine residues of RRM2 or RRM3 resulted in the production of proteins that were less affected by oxidation or reduction. Simultaneous change of cysteines to serines in both RRM2 and RRM3 resulted in the production of a protein that was insensitive to redox regulation. These mutations did not, however, affect the ability of RB47 to bind to the psbA mRNA at high affinity. PABP from *S. cerevisiae* inherently lacks cysteine residues in RRM2 and RRM3, and as shown in Fig. 2, this PABP lacks the ability to be regulated in a redox-dependent manner. Sequence alignment of RB47 and other PABPs suggest that RNA binding activity of other PABPs with cysteines in RRM2 or RRM3 may be redox-regulated in a manner similar to RB47. However, in *vitro* or *in vivo* data supporting this hypothesis are not presently available. The fact that a single mutation in either domain 2 or domain 3 did not produce a protein completely lacking redox regulation suggests that these domains can form disulfide bonds with either domain 1 or 4 in addition to disulfide bonding with each other. This suggests that a cysteine in either domain 2 or 3 along with the conserved cysteines in domain 4 or domain 1 should be sufficient for redox regulation of a PABP.

The three-dimensional structure of domains 1 and 2 of the human PABP has been determined (9). This analysis showed that each RRM forms a four-stranded antiparallel β-sheet with two α-helices packed on one side of the β-sheet (9–11). From this model the cysteine residue of RRM2 is predicted to be located in the β2 sheet and the cysteine residue of RRM3 is located in the α2 helix (10, 12, 16–18). The high conservation among PABPs allows us to model the structure of RB47 using the structure of the human PABP RM1 and RRM2 as a template (9). Because all four RRMs have sequence conservation we were able to model RRM3 and RRM4 along with RRM1 and RRM2 as a complete protein-RNA complex (Fig. 5). We propose the following mechanism of regulation. When RB47 is oxidized, RRM3 folds over onto RRM2 and a disulfide bond between Cys 143 and Cys 259 is formed to stabilize this structure. The bonding of Cys 143 with Cys 259 would create a compacted tertiary structure between RRM2 and RRM3 that would restrict the entry of the psbA mRNA into the RNA binding cleft of the PABP.

The data presented here, taken together with previous characterization of RB47, suggest a model for the light-regulated binding of RB47 to the psbA mRNA. Redox potential generated by the light reactions of photosynthesis is donated to chloroplast protein-disulfide isomerase, which catalyzes the formation and breakage of disulfide bonds between the second and third RRM domains of RB47. Formation or reduction of disulfide bonds changes the conformation of RB47 to inhibit or allow the binding of the psbA mRNA by altering the availability of the RNA binding sites within RB47. We imagine that translation is activated in the chloroplast by the binding of RB47 in much the same way that translation is activated in the cytoplasm by PABP binding, by facilitating the interaction of translation initiation factors with the mRNA and with ribosomal subunits. This mechanism of translational activation, by altering the ability of a PABP to bind to an mRNA by changing sulfide bond status within the protein, may be a general mechanism of translational regulation that functions within many organisms, but one that has yet to be appreciated in other organisms.

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