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Light-regulated Translation of Chloroplast Reaction Center Protein D1 mRNA in *Chlamydomonas reinhardtii*

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Abstract

Light-regulated translation of chloroplast mRNAs requires nuclear-encoded *trans*-acting factors that interact with the 5′ untranslated region (UTR) of these mRNAs. A set of four proteins (60, 55, 47, and 38 kDa) that bind to the 5′-UTR of the *psbA* mRNA had been identified in *C. reinhardtii*. 47 kDa protein (RB47) was found to encode a chloroplast poly(A)-binding protein (cPABP) that specifically binds to the 5′-UTR of the *psb*A mRNA, and essential for translation of this mRNA. cDNA encoding 60 kDa protein (RB60) was isolated, and the amino acid sequence of the encoded protein was highly homologous to plants and mammalian protein disulfide isomerases (PDI), normally found in the endoplasmic reticulum (ER). Immunoblot analysis of *C. reinhardtii* proteins showed that anti-PDI recognized a distinct protein of 56 kDa in whole cell extract, whereas anti-rRB60 detected a 60 kDa protein. The ER-PDI was not retained on heparin-agarose resin whereas RB60 was retained. *In vitro* translation products of the RB60 cDNA can be transported into *C. reinhardtii* chloroplast *in vitro*. Immunoblot analysis of isolated pea chloroplasts indicated that higher plant also possess a RB60 homolog. *In vitro* RNA-binding studies showed that RB60 modulates the binding of cPABP to the 5′-UTR of the *psbA* mRNA by reversibly changing the redox status of cPABP using redox potential or ADP-dependent phosphorylation. Site-directed mutagenesis of -CGHC- catalytic site in thioredoxin-like domain of RB60 demonstrated that the active site of thioredoxin-like domain is responsible for redox-regulated binding of cPABP to the *psbA* mRNA. These results showed that RB60 is an unique PDI located in the chloroplast of *C. reinhardtii*, and suggest that the chloroplast PDI may have evolved to utilize the redox-regulated thioredoxin like domain as a mechanism for regulating the light-activated translation of the *psbA* mRNA.

The D1 protein of photosystem II (PSII) constitutes a D1/D2 heterodimer reaction center core that mediates a primary charge separation. The D1 protein is encoded by the chloroplast *psbA* mRNA, and turns over in the light at a high rate due to the photochemistry within the reaction center, and thus replenishment of this protein into PSII by protein synthesis in the light is necessary to maintain PSII activity [1]. This light-induced synthesis of the D1 protein is regulated at the level of translation [2, 3, 4, 5]. An increase in translation initiation has been shown to be a primary mechanism for light-activated translation of D1 protein in the green algae *Chlamydomonas reinhardtii* and in higher plants [6, 7, 8]. Site-directed mutagenesis of the *C. reinhardtii psbA* mRNA has shown that a structured RNA element contained within the 5′-untranslated region (UTR) is required for the light-activated translation of *psbA* mRNA [8]. Analysis of nuclear mutants of *C. reinhardtii* lacking translation of the *psbA* mRNA has shown that nuclear-encoded *trans*-acting factors are required for the translation of this mRNA [9, 10]. Furthermore, in mutants lacking the RB47 protein, *psbA* mRNA fails to load onto polysomes, and *psbA* mRNA translation and *psbA* mRNA binding activity are not detectable [10]. These results suggest that the interaction of the nuclear-encoded factors with the 5′-UTR of *psbA* mRNA is essential for light-activated translation. The 5′-UTRs of other chloroplast mRNAs have also been identified as key components of translation for these mRNAs [11, 12, 13].

We have previously identified a set of four proteins (60, 55, 47, and 38 kDa) that bind to the 5′-UTR of the *psbA* mRNA in *C. reinhardtii* by *psbA* RNA-specific affinity chromatography [14]. The binding of these proteins to the 5′-UTR of the *psbA* mRNA correlates with the light-activated translation of this mRNA and can be regulated *in vitro* in response to both redox potential and phosphorylation [15, 16]. Recently, cDNA was isolated encoding a member of the *psbA* RNA binding protein complex, the 47 kDa RNA binding protein (RB47) [17]. The encoded RB47 protein is highly homologous to eucaryotic poly(A)-binding proteins (PABP) [17]. The recombinant RB47 protein binds with high specificity to the 5′-UTR of *psbA* mRNA. *In vitro* translation products

of the RB47 RNA were shown to be translocated into the chloroplast and processed to the mature 47 kDa protein. Nuclear mutants of *C. reinhardtii* that lack the RB47 protein have no detectable *psbA* mRNA translation, *psbA* mRNA fails to associate with ribosomes, and no *psbA* specific RNA-binding activity is observed [18]. PABP is known to associate with the 3'-poly(A) tail of cytoplasmic mRNAs and also with the eucaryotic translation initiation factor eIF4G, to stimulate translation initiation [19, 20, 21, 22]. These results suggest a role for RB47 as an activator of *psbA* mRNA translation at the initiation step.

Here we show that RB60 protein is highly homologous to protein disulfide isomerase (PDI), and is capable of regulating the binding of RB47 to the 5'-UTR of the *psbA* mRNA through redox potential and ADP-dependent phosphorylation [23]. PDI is known as a multifunctional protein possessing enzymatic activities for the formation, reduction, and isomerization of disulfide bonds during protein folding and is typically found in the lumen of the ER in mammalian cells. Despite high sequence homology with plant and mammalian PDIs and the presence of the C-terminal -KDEL- ER retention signal, RB60 is a distinct protein from PDI of the ER, and is localized in the stroma of chloroplast. This is a first report on subcellular localization of PDI outside the ER, whose role is to regulate gene expression by modulating RNA binding activity of a RNA specific binding protein.

Molecular Cloning of RB60

To clone the cDNA encoding the 60 kDa *psbA* mRNA binding protein (RB60), the *psbA* specific RNA binding proteins were purified from light-grown *C. reinhardtii* cells using heparin-agarose chromatography followed by *psbA* RNA affinity chromatography (RAC). RAC-purified proteins were separated by two-dimensional polyacrylamide gel electrophoresis, the RB60 protein was digested with trypsin, and unambiguous amino acid sequences were obtained from two peptide fragments. The DNA corresponding to one peptide of 22 amino acid residues was amplified by PCR using degenerate oligonucleotides, and used to screen a λ-gt10 cDNA library from *C. reinhardtii.* The predicted amino acid sequence of the cloned cDNA contained the complete amino acid sequences of the two tryptic peptides. Genomic DNA restricted with several restriction enzymes, and using an RB60 cDNA as a probe at low- (A) and high- (B) stringency conditions, shows that RB60 is encoded by a single gene in the *Chlamydomonas* genome. Additional specific bands were detected by the DNA blot analysis at low-stringency, suggesting that other genes with homology to the RB60 cDNA exist in the *Chlamydomonas* genome. Northern blot analysis identified a single transcript of 2.5 kb encoding the RB60 protein. The predicted amino acid sequence of the encoded protein revealed that it has high sequence homology to both plant and mammalian protein disulfide isomerase (PDI), and contains the highly conserved thioredoxin-like domains with -CysGlyHisCys- (-CGHC-) catalytic sites in both the N-terminal and C-terminal regions and the -LysAspGluLeu- (-KDEL-) endoplasmic reticulum (ER) retention signal at the C-terminus found in all PDIs. PDI is a mutifunctional protein possessing enzymatic activities for the formation, reduction, and isomerization of disulfide bonds during protein folding, and is typically found in the ER [24-26]. The first 30 amino acid residues of RB60 were found to lack sequence homology with the N-terminal signal sequence of PDI from plants or mammalian cells. However, this region has characteristics of chloroplast transit peptides of *C. reinhardtii*, which have similarities with both mitochondrial and higher plant chloroplast presequences [27, 28]. A transit peptide sequence should override the function of the -KDEL- ER retention signal and target the protein to the chloroplast since the -KDEL- signal acts only to retain the transported protein in the ER [24, 25].

The precursor form of RB60 was expressed in *E. coli* as a fusion protein with a (His)10 tag, purified on a Ni-NTA agarose column and analyzed by immunoblotting. A specific band at approximately 64 kDa was detected by antiserum generated against RB60 from *C. reinhardtii*, whereas no signal was detected in protein extracts from *E. coli* cells harboring an expression plasmid without an insert. Purification of the expressed protein by Ni-NTA agarose chromatography resulted in the isolation of a major protein at 64 kDa that strongly reacted with the *C. reinhardtii* RB60 antiserum.

Localization of RB60 Protein into the Chlorplasts

To show that RB60 is a member of the *psbA* RNA binding complex and is localized to the chloroplast, chloroplasts were isolated from a cell wall deficient strain of *C. reinhardtii* (cw15) by percoll gradient centrifugation, and a protein import assay was carried out. Isolated chloroplasts were incubated in the light in the presence of ATP with *in vitro* translated RB60 protein labeled with the [^{35}S] methionine, and then treated with the protease thermolysin to remove the proteins remaining outside the plastids. The samples were then diluted 60-fold with extraction buffer, pelleted and the supernatant removed. The pelleted chloroplasts were analyzed by SDS-PAGE. A prote-

ase-protected radiolabeled RB60 was detected in isolated chloroplasts by this assay. When the pelleted chloroplasts were lysed by treatment with 2% Triton X-100 and then incubated with thermolysin, the radiolabeled imported RB60 was degraded. Addition of 10 mM EDTA, a chelating agent that inhibits thermolysin activity, to the lysed chloroplasts resulted in RB60 protein remaining intact. These data demonstrate that the RB60 protein is imported into the *C. reinhardtii* chloroplast and protected from thermolysin degradation. Similar results were obtained using isolated pea plastids.

To further verify that RB60 is localized to the chloroplasts, an immunoblot analysis of isolated pea chloroplasts was performed using the *C. reinhardtii* RB60 antiserum. To confirm that the isolated pea chloroplasts were free of cytoplasmic contamination, immunoblot analysis was performed with antiserum against the large subunit of ribulose bisphosphate carboxylase (RuBPCase, located in chloroplast) and antiserum against the cytoplasmic protein tubulin. RuBPCase antiserum recognized proteins from both whole leaf extracts (cytoplasm plus chloroplast) and from isolated chloroplasts. The tubulin antiserum recognized a protein in whole leaf extracts, but not in the chloroplast fraction, showing that the isolated chloroplasts were free of cytoplasmic proteins. The protein extracts from isolated pea chloroplasts were enriched using heparin-agarose chromatography: enrichment was required for immunoblot assays with the RB60 antiserum as RB60 is a minor component within the chloroplast. Immunoblot analysis was performed on proteins from purified pea chloroplasts, from *C. reinhardtii* cell extracts isolated by heparin-agarose chromatography, and on recombinant RB60. A specific signal immunochemically related to RB60 was clearly detected at approximately 63 kDa in the pea chloroplast sample. A signal of equal intensity was observed for *C. reinhardtii* proteins and for the recombinant RB60.

RB60 Protein is Distinct from ER-Protein Disulfide Isomerase

PDI is a catalyst that assists in folding of proteins containing disulfide bonds in the endoplasmic reticulum [26]. PDI enzymes and the encoding cDNAs have been isolated from a variety of species of mammals and plants [26]. *C. reinhardtii* PDI has been purified to near homogeneity by a series of column chromatography steps using a conventional PDI enzyme assay, and antiserum against this authentic PDI has been generated [29]. In order to determine whether RB60 is the same PDI functioning in the ER or a different protein, an immunoblot analysis was performed using antisera to both rRB60 (anti-rRB60) and authentic PDI (anti-PDI) purified from *C. reinhardtii*. Proteins from whole cell extracts of *C. reinhardtii*, or proteins isolated by heparin-agarose chromatography, or purified rRB60 protein were separated on SDS-PAGE, transferred to membrane, and reacted with anti-rRB60 and anti-PDI. Anti-rRB60 recognizes a 60 kDa protein from both whole cells and from proteins isolated by heparin-agarose chromatography, as well as the rRB60 protein. Anti-PDI recognized a distinct protein of 56 kDa in whole cell extracts. Anti-PDI recognized a 60 kDa protein (RB60) from the heparin-agarose purified proteins and the rRB60 protein, due to cross-immuno reactivity of the related RB60 protein when it is in a concentrated form as it is in these two fractions. The anti-PDI did not recognize a protein corresponding to the 56 kDa ER-PDI, indicating that ER-PDI was not retained on the heparin-agarose resin. These data clearly show that RB60 is a distinct protein from PDI in *C. reinhardtii*, with a different size mass and differential reactivity with anti-rRB60 and anti-PDI. Fractionation of chloroplasts from cell wall-deficient*C. reinhardtii* showed that RB60 is present mainly in the stroma. These results are in good agreement with the Southern blot analysis, that shows that RB60 is encoded by a single gene, but that also shows that other related DNA sequences, probably encoding the ER-PDI, are also identified with the RB60 cDNA probe.

RB60 Protein Catalyzes Redox-Regulated Binding of RB47 to the psbA *mRNA*

Chloroplast PDI (cPDI) contains the two -CGHC- catalytic sites that are involved in the formation, reduction and isomerization of disulfide bonds associated with protein folding. The identification of these redox catalytic sites prompted us to investigate the role of RB60 in the redox-regulated binding of RB47 to the 5′-UTR of the *psbA* mRNA. The endogenous form of RB47 containing only the four RNA recognition motif domains was expressed in *E. coli* as a fusion protein with a $(His)_{10}$ tag, purified on a Ni-NTA agarose affinity column and used for subsequent RNA binding gel mobility-shift assays. We first investigated whether RNA binding activity of recombinant RB47 (r-RB47) could be altered by the addition of a reducing agent (DTT, dithiothreitol) in the presence of recombinant RB60 (r-RB60). r-RB47 was preincubated with 10 mM DTT, a 5-fold excess of r-RB60 alone, or both DTT plus r-RB60, prior to adding a ^{32}P-labeled 5′-UTR of the *psbA* mRNA, followed by a gel mobility-shift assay. These data showed that r-RB47 isolated from *E. coli* is in an

active reduced form so that only a slight enhancement of RNA binding activity could be obtained with addition of DTT and r-RB60. To determine whether r-RB60 is able to re-activate r-RB47 that is in an inactive oxidized form, r-RB47 was incubated with the oxidant dithionitrobenzoic acid (DTNB) for 5 min and then dialyzed against 104 volume of buffer to remove the oxidant. Oxidation of r-RB47 by DTNB completely abolished the binding activity of the protein. Addition of DTT to 1.0 mM partially restored the binding capacity of r-RB47, and the binding can be increased three fold by the addition of up to 25 mM DTT. With increasing amounts of r-RB60, the binding activity of r-RB47 was increased compared to the samples without r-RB60 at every level of DTT tested. When DTT was not present in the incubation medium, r-RB60 alone could not restore the binding of the oxidized r-RB47 (0 mM DTT), indicating that r-RB60 requires reducing equivalents to convert the inactive oxidized form of r-RB47 to an active reduced form.

Protein disulfide isomerase is known to catalyze the formation of disulfide bonds by oxidation of the sulfhydryl groups of cysteine residues during protein folding. To examine whether r-RB60 is also capable of oxidative catalysis of the reduced form of r-RB47, GSSG, the oxidized form of the thiol tripeptide glutathionine, was added to the assay mixture. When GSSG alone was added to r-RB47 at up to 5 mM, there was a two fold reduction in binding activity of r-RB47 compared with untreated protein. Incubation of r-RB47 with both GSSG and r-RB60 reduced the binding activity of r-RB47 by 5-6 fold, indicating that r-RB60 can facilitate the conversion of the reduced form of r-RB47 to an inactive oxidized form under an oxidizing environment.

Active Site of Thioredoxin-like Domain in RB60 is Responsible for Redox-Regulated RNA-Binding of RB47

Two thioredoxin-like domains of RB60 contain the most highly conserved motif common to the a and a' domains of PDI, the sequence EFYAPWCGHCK that aligns with *E. coli* thioredoxin sequence DFWAEWCGPCK. Thioredoxin functions as a thiol-disulfide redox protein, and the Cys residues within this sequence are the redox-active groups. Earlier chemical modification experiments and mutagenesis studies clearly implicate that the first Cys residue within this homologous sequence functions as the redox-active groups at the active sites of PDI, and a and a' domains function independently in redox-catalysis [30, 31]. To investigate whether –CGHC-motif of RB60 functions as active site in redox-regulated RNA binding of RB47, the first Cys residue was mutagenized to Ser in each of thioredoxin-like domain and in both N- and C-terminal domains. Mutant RB60 proteins were then expressed as His_{10} tag-fusion proteins in *E. coli* and purified on Ni-NTA column. Each mutant RB60 protein was incubated with rRB47 oxidized with DTNB, in the presence of 0.1mM DTT and added with a ^{32}P-labeled 5′-UTR of the *psbA* mRNA, followed by gel-mobility shift assay. When first Cys residue of each thioredoxin-like domain active site was changed to Ser, the re-activation of RNA-binding activity of RB47 was similar to that of RB47 by wild type RB60. However, double mutations of first Cys residues of two active sites in RB60 did not cause the re-activation of the RNA-binding activity of oxidized RB47 by double mutant RB60. These results demonstrate that –CGHC- catalytic active sites of thioredoxin-like domains are essential for the redox-regulated RNA binding of RB47 by RB60, and each domain functions independently and enough for full catalytic activity.

RB60 Reduces the psbA *RNA-Binding of RB47 Through ADP-Dependent Phophorylation*

ADP-dependent phosphorylation of RB60 has previously been shown to reduce binding of the protein complex to the 5′-UTR of the *psbA* mRNA [15]. To identify if recombinant RB60 can be phosphorylated, r-RB60 was incubated with heparin-purified proteins from *C. reinhardtii* in the presence of γ-32P-ATP. Phosphorylated r-RB60 was detected among a number of phosphorylated proteins in the heparin-purified fraction. Purification of the incubation mixtures on Ni-NTA resin resulted in the isolation of phosphorylated r-RB60 as indicated by the arrow. Phosphorylated r-RB60 was able to reduce the binding of r-RB47 to the 5′-UTR of the *psbA* mRNA whereas, phosphorylated *C. reinhardtii* proteins eluted from Ni-NTA resin had little impact on r-RB47 RNA binding.

A Working Model

It has previously been shown that thioredoxin can act as a transducer of redox potential to enhance the binding of a protein complex to the *psbA* mRNA [16]. PDI fits well into this scheme as ferredoxin-thioredoxin reductase is capable of directly reducing PDI [32, 33]. We propose that reducing equivalents donated to cPDI through ferredoxin and ferredoxin-thioredoxin reductase act to catalyzes the reduction of chloroplast poly(A)-binding protein (cPABP). The reduced form of cPABP is then capable of binding to

the 5′-UTR of the *psbA* mRNA to activate translation initiation of this mRNA resulting in increased synthesis of the D1 protein. Protein disulfide isomerase has an additional advantage in this scheme in that it has greater oxidation potential than thioredoxin [26], thus allowing the off switch (oxidation) when reducing potential is low. ADP-dependent phosphorylation of RB60, that might be triggered by the increased pool of ADP during dark growth, can act to reduce the RNA binding activity of cPABP by enhancing the oxidative catalysis of cPDI over the reductive catalysis, resulting in decreased translation of the *psbA* mRNA.

The chloroplast form of PDI may have evolved to utilize a redox-regulated thioredoxin-like domain for the light-activated binding of RB47 to the *psbA* mRNA. The binding of RB47 to the 5′ UTR of the *psbA* mRNA is used to regulate translation of this chloroplast mRNA upon light exposure. We imagine that cPDI is also used for a number of other redox reactions within the chloroplast, and that regulation of RB47 binding and *psbA* mRNA translation is only one of many events that are regulated in the chloroplast by cPDI as a means to achieve redox control of key chloroplast functions. For example, splicing of *psbA* pre-RNA in *Chlamydomonas* is redox-regulated [34] and a 60 kDa protein is involved in the splicing of *psbA* RNA. The binding of proteins to the 3′end of chloroplast mRNAs is regulated by 60 kDa protein using ADP-dependent phosphorylation. ADP-dependent phosphorylation was also shown to regulate the association of a sequence specific DNA-binding protein complex with the barley chloroplast *psbD* blue-light-responsive promoter [35]. It is likely that RB60 protein homolog, a cPDI, in barley chloroplast modulates the binding of protein complex to the *psbD* promoter. Given the fact that genetic information between the nucleus and the chloroplast is exchanged [36], the utilization of PDI, a normally ER localized enzyme, as a regulator in the chloroplast seems reasonable. Many chloroplast proteins are nuclear-encoded, synthesized in the cytoplasm and post-translationally transported into the chloroplast [37]. The KDEL retention sequence contained at the C-terminus of RB60 may be a vestige identifying the origin of the RB60 gene (from the ER-PDI gene) that is retained in the chloroplast protein simply because there is no selective pressure to remove it, as KDEL sequences act only as retention signals not as targeting sequences [24].

It has been suggested that PDI is a single mutifunctional protein possessing a variety of specificities and activities [38]. However, growing evidence shows that there are a number of cDNA sequences that encode proteins that resemble PDI in having thioredoxin-like domains, each containing the CGHC motif of the active site of PDI [26]. PDI has also shown to be the β-subunit of prolyl hydroxylase, an enzyme that catalyzes the post-translational hydroxylation of peptidyl proline residues within pro-β-chains of procollagen [38]. Microsomal preparations from mammalian tissues contain a PDI identified as a component of the complex that catalyzes the transfer of triglyceride and cholesteryl esters between membranes [39]. This diversity of PDI family members suggests that there must be a number of functional differentiations, and possibly differences in subcellular distribution and substrate range, for this enzyme. The results presented here show that *in C. reinhardtii* the RB60 protein has functionally differentiated from an ancestoral ER localized PDI that functions as a folding catalyst, and has moved to the chloroplast where it acts as a redox regulator of key chloroplast enzymatic funtions.

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