Protein Disulfide Isomerase as a Regulator of Chloroplast Translational Activation

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Light-regulated translation of chloroplast messenger RNAs (mRNAs) requires *trans*-acting factors that interact with the 5′ untranslated region (UTR) of these mRNAs. Chloroplast polyadenylate-binding protein (cPABP) specifically binds to the 5′-UTR of the *psbA* mRNA and is essential for translation of this mRNA. A protein disulfide isomerase that is localized to the chloroplast and copurifies with cPABP was shown to modulate the binding of cPABP to the 5′-UTR of the *psbA* mRNA by reversibly changing the redox status of cPABP through redox potential or adenosine 5′-diphosphate–dependent phosphorylation. This mechanism allows for a simple reversible switch regulating gene expression in the chloroplast.

Synthesis of certain chloroplast photosynthetic proteins is activated 50- to 100-fold in response to light exposure without an increase in the corresponding mRNA levels, indicating that translation of chloroplast mRNAs is light-regulated (*1*). Genetic evidence has shown that nuclear-encoded *trans*-acting factors interact with the 5′-UTR of chloroplast mRNAs to activate translation of these mRNAs in a light-dependent manner (*2*, *3*). A set of proteins (38, 47, 55, and 60 kD) was identified that binds as a complex to the 5′-UTR of the *psbA* mRNA, encoding the photosynthetic reaction center protein D1 from the green algae *Chlamydomonas reinhardtii* (*3*–*6*). Binding of this protein complex to the 5′-UTR of the *psbA* mRNA correlates with light-enhanced translation of this mRNA under a variety of environmental conditions and in mutations deficient in *psbA* mRNA translation (*4*–*8*). RNA binding activity of the protein complex for the 5′-UTR of the *psbA* mRNA can be regulated in vitro by at least two different mechanisms: adenosine 5′-diphosphate (ADP)–dependent phosphorylation and changes in redox potential (*7*, *9*). Recently, a cDNA encoding the 47-kD RNA binding protein (RB47) was cloned that binds specifically to the 5′-UTR of *psbA* mRNA from *C. reinhardtii* chloroplast. The nuclear-encoded protein is homologous to PABP and is translocated to the chloroplast (*8*). Biochemical analysis of *C. reinhardtii* mutants lacking *psbA* mRNA translation shows that both RB47 and *psbA*-specific RNA binding activity are required for *psbA* mRNA translation (*8*).

To clone the cDNA encoding the 60-kD *psbA* mRNA binding protein (RB60), we purified the *psbA*-specific RNA binding pro-

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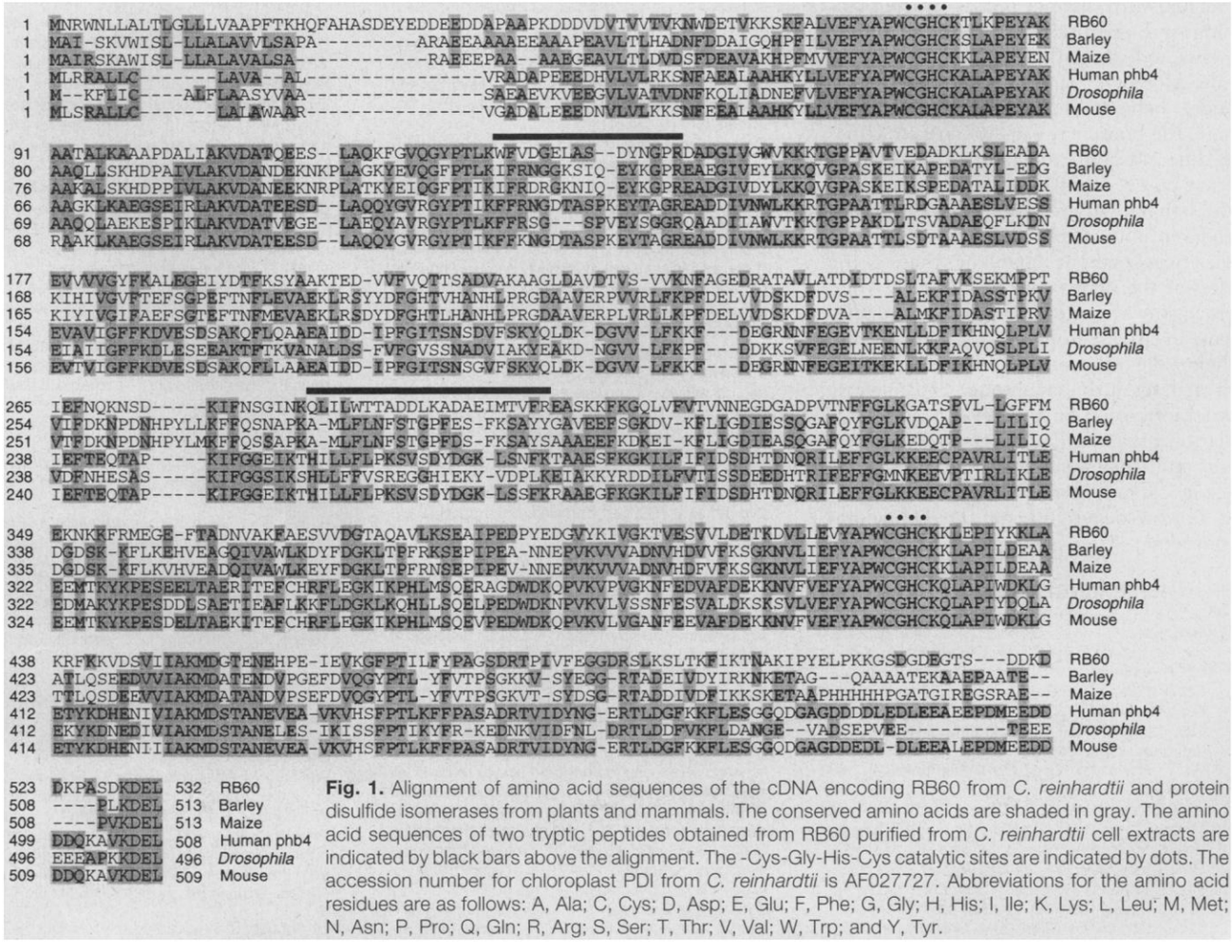

Fig. 1. Alignment of amino acid sequences of the cDNA encoding RB60 from *C. reinhardtii* and protein disulfide isomerases from plants and mammals. The conserved amino acids are shaded in gray. The amino acid sequences of two tryptic peptides obtained from RB60 purified from *C. reinhardtii* cell extracts are indicated by black bars above the alignment. The -Cys-Gly-His-Cys catalytic sites are indicated by dots. The accession number for chloroplast PDI from *C. reinhardtii* is AF027727. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

teins 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 (PAGE), the RB60 protein was digested with trypsin, and unambiguous amino acid sequences were obtained from two peptide fragments (*10*). The DNA corresponding to one peptide of 22 amino acid residues was amplified by polymerase chain reaction with 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 (Fig. 1). The 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 -Cys-Gly-His-Cys- (-CGHC-) catalytic sites in both the NH_2- and COOH-terminal regions (Fig. 1) and the -Lys-Asp-Glu-Leu- (-KDEL-) endoplasmic reticulum (ER) retention signal at the COOH-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 (*11–14*). The first 30 amino acid residues of RB60 were shown to lack sequence homology with the NH_2-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 (*15, 16*). A transit peptide sequence should override the function of the -KDEL- ER retention signal and target the protein to the chloroplast because the -KDEL- signal acts only to retain the transported protein in the ER (*11–13*).

To verify that RB60 is localized to the chloroplasts, we performed an immunoblot analysis of isolated pea chloroplasts using the C. *reinhardtii* antiserum to RB60 (anti-RB60) (*17*). To confirm that the isolated pea chloroplasts were free of cytoplasmic contamination, we performed immunoblot analysis with antiserum against the large subunit of ribulose bisphosphate carboxylase (RuBPCase, located in the chloroplast) and antiserum against the cytoplasmic protein tubulin. Anti-RuBPCase recognized proteins from both whole-leaf extracts (cytoplasm plus chloroplast) and from isolated chloroplasts (Fig. 2A, lanes 3 and 4). Anti-tubulin recognized a protein in whole-leaf extracts (lane 5, arrow), but not in the chloroplast fraction (lane 6), showing that the isolated chloroplasts were free of cytoplasmic proteins. The protein extracts from isolated pea chloroplasts were enriched by heparin-agarose chromatography; enrichment was required for immunoblot assays with anti-RB60 because RB60 is a minor component within the chloroplast (*4*). 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 detected at ~63 kD in the pea chloroplast sample (Fig. 2B, lane 4). A signal of equal intensity was observed for C. *reinhardtii* proteins and for the recombinant RB60 (Fig. 2B, lanes 5 and 6).

Chloroplast PDI (cPDI) contains the two -CGHC- catalytic sites that regulate the formation, reduction, and isomerization of disulfide bonds associated with protein folding (Fig. 1). 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 (*7*). The precursor form of RB60 and the endogenous form of RB47, containing only the four RNA recognition motif domains (*18*), were expressed in *Escherichia coli* as a fusion protein with a $(His)_{10}$ tag, purified on a Ni–nitrilotriacetic acid (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, dithiothreitol (DTT), in the presence of recombinant RB60 (r-RB60). r-RB47 was preincubated with 10 mM DTT, a fivefold excess of r-RB60 alone, or both DTT and r-RB60 before addition of a ^{32}P-labeled 5′-UTR of the *psbA* mRNA, followed by a gel mobility-shift assay (Fig. 3A). These data showed

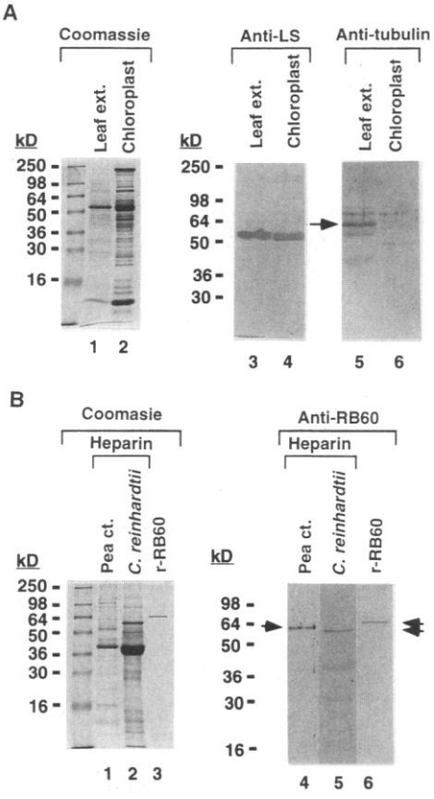

Fig. 2. Localization of RB60 to chloroplasts. (**A**) The protein samples from whole-leaf extracts (lanes 1, 3, and 5) and isolated pea chloroplast (lanes 2, 4, and 6) (*23*) were separated by SDS-PAGE and either stained with Coomassie blue (lanes 1 and 2) or analyzed by immunoblotting with antiserum to RuBPCase (lanes 3 and 4) or antiserum to cytoplasmic tubulin (lanes 5 and 6). (**B**) Pea chloroplasts (lane 1), C. *reinhardtii* proteins (lane 2) partially purified by heparin-agarose chromatography (*4, 8*), and r-RB60 (lane 3) were separated by SDS-PAGE and either stained with Coomassie blue (left) or subjected to immunoblot analysis with the C. *reinhardtii* anti-RB60 (right).

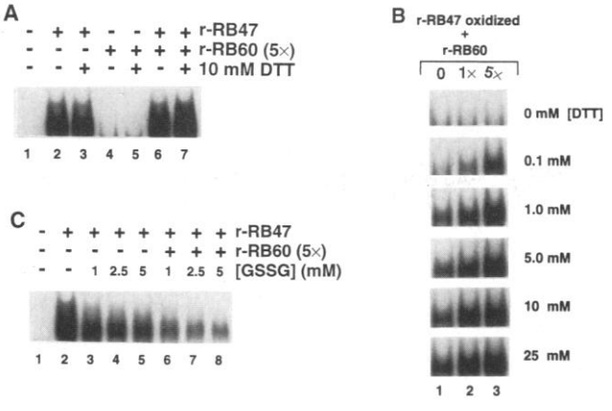

Fig. 3. r-RB60 regulates the binding of chloroplast PABP to the 5′-UTR of *psbA* mRNA through redox equivalents. (**A**) r-RB47 was incubated with 10 mM DTT and r-RB60 as indicated, and then subjected to gel mobility-shift assay with a ^{32}P-labeled 5′-UTR of the *psbA* mRNA. Only the portions of the gel containing the RNA-protein complexes are shown. (**B**) r-RB47 (0.2 μg) oxidized with DTNB was incubated with increasing concentrations of DTT in the presence (lanes 2 and 3) or absence (lane 1) of r-RB60 with a molar ratio of 1:1 (lane 2) or 5:1 (lane 3) over r-RB47. (**C**) r-RB47 (0.2 μg) was incubated with increasing concentrations of GSSG in the presence (lanes 6 to 8) or absence (lanes 3 to 5) of r-RB60. r-RB60 was added in a fivefold molar excess over r-RB47.

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 reactivate r-RB47 that is in an inactive oxidized form, we incubated r-RB47 with the oxidant dithionitrobenzoic acid (DTNB) for 5 min and then dialyzed it against 10^4 volume of buffer to remove the oxidant. Oxidation of r-RB47 by DTNB completely abolished the binding activity of the protein (Fig. 3B). Addition of DTT to 1.0 mM partially restored the binding capacity of r-RB47 (lane 1), and the binding could be increased threefold 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 concentration of DTT tested (lanes 2 and 3). 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.

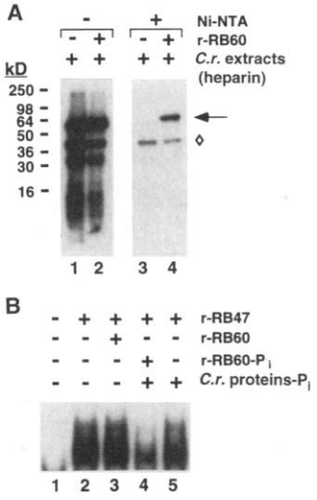

Fig. 4. Decrease in the binding of cPABP to the 5′-UTR of *psbA* mRNA by the addition of phosphorylated r-RB60. (**A**) *Chlamydomonas reinhardtii* proteins were incubated for 20 min at room temperature in the presence of [γ-^{32}P]ATP with (lanes 2 and 4) or without (lanes 1 and 3) the purified r-RB60 protein. Phosphorylated proteins were separated by SDS-PAGE with (lanes 3 and 4) or without (lanes 1 and 2) purification by Ni-NTA resin and autoradiographed. (**B**) r-RB47 (lane 2) was incubated for 20 min at room temperature with r-RB60 (lane 3) or r-RB60 that had been phosphorylated with 5 mM ATP through use of the heparin-purified protein extracts and re-isolated by Ni-NTA resin (lane 4), followed by gel mobility-shift assay.

PDI catalyzes 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, we added GSSG, the oxidized form of the thiol tripeptide glutathionine, to the assay mixture. When GSSG alone was added to r-RB47 at up to 5 mM (Fig. 3C, lanes 3 to 5), binding activity of r-RB47 decreased by a factor of 2 compared with untreated protein (Fig. 3C, lane 2). Incubation of r-RB47 with both GSSG and r-RB60 reduced the binding activity of r-RB47 by a factor of 5 to 6 (Fig. 3C, lanes 6 to 8), indicating that r-RB60 can facilitate the conversion of the reduced form of r-RB47 to an inactive oxidized form under an oxidizing environment.

ADP-dependent phosphorylation of RB60 reduces binding of the protein complex to the 5′-UTR of the *psbA* mRNA (9). To identify if r-RB60 can be phosphorylated, we incubated r-RB60 with heparin-purified proteins from C. *reinhardtii* in the presence of [γ-^{32}P]ATP (adenosine 5′-triphosphate). Phosphorylated r-RB60 was detected (Fig. 4A, lane 2) 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 (Fig. 4A, lane 4). Phosphorylated r-RB60 reduced the binding of r-RB47 to the 5′-UTR of the *psbA* mRNA (Fig. 4B, lane 4) (*19*), whereas phosphorylated C. *reinhardtii* proteins eluted from Ni-NTA resin (Fig. 4A, diamond) had little effect on r-RB47 RNA binding (Fig. 4B, lane 5).

Thioredoxin can act as a transducer of redox potential to enhance the binding of a protein complex to the *psbA* mRNA (*7*). PDI fits well into this scheme because ferredoxin-thioredoxin reductase is capable of directly reducing PDI (*20*, *21*). As shown in a schematic model (Fig. 5), we propose that reducing equivalents, generated by photosynthesis, are donated to cPDI through ferredoxin and ferredoxin-thioredoxin reductase and act to catalyze the reduction of chloroplast polyadenylate-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 (*4*, *8*, *9*, *22*). This mechanism provides a direct link in the chloroplast between the quantity of absorbed light and the rate of synthesis of the D1 protein, allowing the replacement of the photo-damaged D1 protein. PDI has an additional advantage in this scheme in that it has greater oxidation potential than thioredoxin (*14*), thus allowing the off switch (oxidation) when reducing potential is low. ADP-dependent phosphorylation of RB60, which 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 data presented here show that a PDI can act as a regulator of RNA binding activity and hence gene expression, and not just as a catalyst for protein folding.

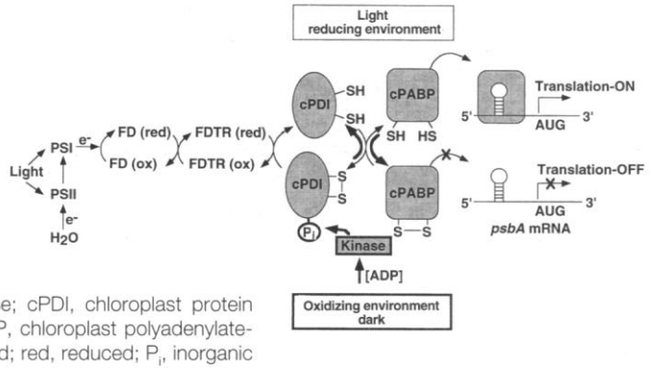

Fig. 5. A working model for light-regulated translation of chloroplast *psbA* mRNA in *C. reinhardtii*. See text for description. Thin arrows and thick arrows indicate the light pathway and the dark pathway, respectively. PSI and PSII, photosystem I and II; FD, ferredoxin; FDTR, ferredoxin-thioredoxin reductase; cPDI, chloroplast protein disulfide isomerase; cPABP, chloroplast polyadenylate-binding protein; ox, oxidized; red, reduced; P_i, inorganic phosphate group.

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17. An immunoblot analysis of isolated chloroplasts from *C. reinhardtii* cells was performed with both antisera to tubulin and *C. reinhardtii* RB60. Although a specific 60-kD band was detected in isolated *C. reinhardtii* chloroplasts, it was not possible to obtain pure chloroplasts free of cytoplasmic proteins by density gradient centrifugation because of the similar density of whole cells and isolated chloroplasts. We therefore used pea chloroplasts for this assay, which can be readily obtained in pure form by Percoll gradient centrifugation.
18. Full-length r-RB47 is 69 kD and contains a COOH-terminal domain that is cleaved in vivo to yield the native 47-kD protein (*8*). To generate the endogenous form of RB47, we mapped the location of the 47-kD polypeptide on the full-length recombinant protein by comparing mass spectrometric data of tryptic digests of the *C. reinhardtii* 47-kD protein with the full-length recombinant protein. These data showed that the 47-kD protein encompasses the four RNA recognition motif domains found in all polyadenylate [poly(A)]-binding proteins, but lacked the COOH-terminal portion of the protein.
19. To examine whether auto-phosphorylation of r-RB60 could affect the binding of r-RB47 to the 5′-UTR of *psbA* mRNA, we incubated r-RB47 and r-RB60 in the presence or absence of 3 mM ATP followed by gel mobility-shift assay. The RNA binding activity of r-RB47 was not altered by the addition of ATP, showing that auto-phosphorylation of r-RB47 or r-RB60 is not responsible for reducing r-RB47 RNA binding.
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Sequence-Specific and Phosphorylation-Dependent Proline Isomerization: A Potential Mitotic Regulatory Mechanism

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Pin1 is an essential and conserved mitotic peptidyl-prolyl isomerase (PPIase) that is distinct from members of two other families of conventional PPIases, cyclophilins and FKBPs (FK-506 binding proteins). In response to their phosphorylation during mitosis, Pin1 binds and regulates members of a highly conserved set of proteins that overlaps with antigens recognized by the mitosis-specific monoclonal antibody MPM-2. Pin1 is here shown to be a phosphorylation-dependent PPIase that specifically recognizes the phosphoserine-proline or phosphothreonine-proline bonds present in mitotic phosphoproteins. Both Pin1 and MPM-2 selected similar phosphorylated serine-proline–containing peptides, providing the basis for the specific interaction between Pin1 and MPM-2 antigens. Pin1 preferentially isomerized proline residues preceded by phosphorylated serine or threonine with up to 1300-fold selectivity compared with unphosphorylated peptides. Pin1 may thus regulate mitotic progression by catalyzing sequence-specific and phosphorylation-dependent proline isomerization.

Proline-directed protein kinases, such as cyclin-dependent protein kinases, play important roles in regulating eukaryotic cell division (*1*). At the G_2-to-M phase transition of the cell cycle, a rapid increase in Cdc2 kinase activity results in phosphorylation of many proteins on serine or threonine residues that are followed by proline (S/T–P sites) (*2*). Phosphorylation of these proteins is thought to trigger many of the structural modifications that occur during mitosis (*1–3*). However, it is not clear how this abrupt change in phosphorylation state leads to an organized and programmed set of mitotic events.

Pin1, originally isolated as a protein that interacts with and inhibits the essential mitotic kinase NIMA, is highly conserved and possesses both a WW domain and PPIase activity (*4*). PPIases catalyze the relatively slow peptidyl-prolyl isomerization of proteins, allowing relaxation of local energetically unfavorable conformational states (*5*). The cyclophilins and FKBPs, two major families of PPIases, are targets of clinically relevant immunosuppressive drugs. However, the immunosuppressive activity of these drugs is unrelated to inhibition of PPIase activity, and neither cyclophilin nor FKBP genes are essential (*5*). Thus, evidence for the biological importance of this enzymatic activity is limited.

In contrast, Pin1, a member of a family of PPIases that differ from cyclophilins and FKBPs, is essential for cell growth and requires a catalytically competent PPIase domain (*4*, *6*). Pin1 both negatively regulates entry into mitosis and is required for normal progression through mitosis in human cells and yeast (*4*). We have recently shown that Pin1 specifically interacts with and regulates the activity of a subset of mitotic proteins in a phosphorylation-dependent manner (*7*). However, little is known about how these specific interactions are achieved.

To investigate the mechanism by which Pin1 interacts with essential mitotic proteins, we used a glutathione S-transferase (GST)–Pin1 fusion protein to screen oriented degenerate peptide libraries (*8*). The crystal structure of Pin1 containing an Ala-Pro dipeptide substrate revealed a sulfate ion located 5 Å from the C_β carbon of Ala, suggesting that phosphorylated Ser (pS) might be preferred at this site (*9*). To test this idea, we prepared a pS-containing degenerate peptide library of general sequence H_2N-MAXXXpSXXXAKK, where X is any amino acid except Cys (*8*). The GST-Pin1 protein preferentially bound a subset of peptides with Pro immediately COOH-terminal to pS (Fig. 1A).

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