Interactions of Arabidopsis RS Domain Containing Cyclophilins with SR Proteins and U1 and U11 Small Nuclear Ribonucleoprotein-specific Proteins Suggest Their Involvement in Pre-mRNA Splicing*§

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Ser/Arg (SR)-rich proteins are important splicing factors in both general and alternative splicing. By binding to specific sequences on pre-mRNA and interacting with other splicing factors via their RS domain they mediate different intraspliceosomal contacts, thereby helping in splice site selection and spliceosome assembly. While characterizing new members of this protein family in Arabidopsis, we have identified two proteins, termed CypRS64 and CypRS92, consisting of an N-terminal peptidyl-prolyl cis/trans isomerase domain and a C-terminal domain with many SR/SP dipeptides. Cyclophilins possess a peptidyl-prolyl cis/trans isomerase activity and are implicated in protein folding, assembly, and transport. CypRS64 interacts in vivo and in vitro with a subset of Arabidopsis SR proteins, including Srp30 and Srp34 homologs of mammalian SP2/ASF, known to be important for 5’ splice site recognition. In addition, both cyclophilins interact with U1-70K and U11-35K, which in turn are binding partners of Srp34/SR1. CypRS64 is a nucleoplasmic protein, but in most cells expressing CypRS64-GFP fusion it was also found in one to six round nuclear bodies. However, co-expression of CypRS64 with its binding partners resulted in re-localization of CypRS64 from the nuclear bodies to nuclear speckles, indicating functional interactions. These findings together with the observation that binding of Srp34/SR1 to CypRS64 is phosphorylation-dependent indicate an involvement of CypRS64 in nuclear pre-mRNA splicing, possibly by regulating phosphorylation/dephosphorylation of SR proteins and other spliceosomal components. Alternatively, binding of CypRS64 to proteins important for 5’ splice site recognition suggests its involvement in the dynamics of spliceosome assembly.

Most plant and metazoan genes are interrupted by introns, which have to be removed from pre-mRNAs in the splicing process. Pre-mRNA splicing takes place in the spliceosome, a large ribonucleoprotein that assembles anew on each intron from five small nuclear ribonucleoprotein (snRNP) particles binding numerous additional proteins. Fully assembled, catalytically active spliceosome contains at least 200 different proteins, making the spliceosome the most complex cellular machine characterized so far (1, 2). Spliceosome assembly is a highly ordered and dynamic process involving many structural rearrangements. The snRNPs, with assistance of the non-snRNP proteins, assemble onto the pre-mRNA intron in a coordinated manner. In the early spliceosomal complex U1 and U2 snRNPs are required for the initial definition of 5’ and 3’ splice sites, respectively. A mature spliceosome is formed by the U4/U6.U5 tri-snRNP, which finally leads to the displacement of U1 and U4 snRNPs (reviewed in Refs. 3–5). During this process several snRNA-snRNA and snRNA-mRNA interactions are disrupted, and others are formed. Proteins that are involved in these RNA rearrangements have been characterized and are known as RNA helicases (reviewed in Refs. 6 and 7).

One important family of splicing factors consists of serine/arginine (SR) proteins, which are required for intron recognition and spliceosome assembly. They are composed of one or two N-terminally positioned RNA recognition motifs, interacting with specific sequences in the pre-mRNA, and a C-terminal RS domain, involved in protein-protein interaction. These two activities of SR proteins are important for promoting various intraspliceosomal contacts (reviewed in Refs. 8–10). Binding of SR proteins to exonic (intronic) splicing enhancers (silencers) helps in recruitment of U1 snRNP to the 5’ splice site and U2 snRNP to the branch point sequence. The first reaction is mediated by interaction of SR proteins with the U1 snRNP-specific protein U1–70K. Interactions of SR proteins with U2AF35, the small subunit of the heterodimeric U2AF (which is important for definition of the 3’ splice site), stabilize U2AF at the polypyrimidine tract. This is important for efficient splicing of U2 snRNP binding to the polypyrimidine tract, which is necessary for the progression of spliceosome assembly (reviewed in Refs. 3–5, 10). SR proteins are phosphoproteins, and their phosphorylation status can regulate their ability to interact with other splicing factors (reviewed in Refs. 8 and 9). In addition, it has also been shown that SR proteins play an important role in splicing of minor, AT-AC class introns (11).

The protein composition of the assembling spliceosome is also dramatically changing, and many proteins found in early

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The abbreviations used are: snRNP, small nuclear ribonucleoprotein; CB, Cajal body; PPIase, peptidyl-prolyl cis/trans isomerase; HA, hemagglutinin; GST, glutathione S-transferase; GFP, green fluorescent protein; RFP, red fluorescent protein; CIP, calf intestinal phosphatase; PEB, protoplast extraction buffer.

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steps of spliceosomal assembly are not present in catalytically active spliceosomes. The driving force for the formation and disruption of specific protein-protein and protein-RNA interactions is not known, but kinases, phosphatases, and cyclophilins are likely candidates. Cyclophilins are a large family of proteins present in all three major phyla. Moreover, in eukaryotic cells they have been found in most cellular compartments, including the cytosol, endoplasmic reticulum, mitochondria, chloroplasts, and the nucleus (reviewed in Refs. 12–14). The peptidyl-prolyl cis/trans isomerase (PPIase) or cyclophilin domain (~160 amino acids) is highly conserved in all members of this protein family, and individual proteins differ mainly in their N- and/or C-terminal extensions. Because of their peptidyl-prolyl cis/trans isomerase activity in vitro, they have been thought to play an important role in protein folding. In addition, they have been proposed to act as molecular chaperones, by binding to protein sequences containing prolines. Indeed, some cyclophilins have been found in transient or stable complexes with specific proteins (reviewed in Refs. 12–14)

Recently, a U4/U6 snRNP-specific protein, termed USA-Cyp or SnuCyp-20, was identified as a cyclophilin (15, 16). In human cells, USA-Cyp forms stable complexes with hPrp3, hPrp4, or SnuCyp-20, was identified as a cyclophilin (15, 16). In human cells, USA-Cyp forms stable complexes with hPrp3, hPrp4, or SnuCyp-20, which was proposed to act as molecular chaperone, by binding to protein sequences containing prolines. Indeed, some cyclophilins have been found in transient or stable complexes with specific proteins (reviewed in Refs. 12–14).

In a yeast two-hybrid screen with Arabidopsis SR proteins SCL28, SCL30, and SRP30, we found an interaction with a protein containing a PPIase domain and a C-terminal domain containing many SR repeats (18–21). A yeast two-hybrid screen with Arabidopsis SR proteins SCL28, SCL30, and SRP30, we found an interaction with a protein containing a PPIase domain and a C-terminal domain containing many SR repeats (18–21). In contrast to USA-Cyp, they are large proteins consisting of an N-terminal PPIase domain and a C-terminal domain containing many SR repeats (18–21).

Plant cells express a complex set of SR proteins (22). Some of them seem to be plant-specific (22), and together with the recently identified intron-binding protein UBP1 (23), they might be responsible for differences in early steps of intron recognition between plants and metazoa (reviewed in Refs. 24 and 25). In a yeast two-hybrid screen with Arabidopsis SR proteins SCL28, SCL30, and SRP30, we found an interaction with a protein containing a PPIase domain and a C-terminal domain. Here, we show that this protein, named CyPRS64, and one additional RS domain-containing cyclophilin, CypRPS92, interact with Arabidopsis SR proteins and with U1 and U11 snRNPs. Specific proteins U1–70K and U1–35K, respectively. We discuss the data in the light of a possible function of both cyclophilins in the dynamic organization of the splicing machinery.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions**—The yeast two-hybrid plasmids, pBD-SCL28, pBD-SCL30, and pBD-SRP30, used for screening were described elsewhere. The yeast vectors used for the expression of hemagglutinin (HA)-tagged SCL28, SCL30, SRP30, and SRP34 have been described (26). The yeast two-hybrid plasmids pBD-CypRS64 and pBD-CypRS92 were created by cloning PCR products obtained with gene-specific primers that introduce EcoRI and Sall restriction sites in front of the start codon and after the stop codon, respectively, into pGBT9 (Clontech). The same PCR fragments were used for construction of glutathione S-transferase (GST) fusions in pGEX-4T-1 (Amersham Biosciences). Five deletion mutants in pGEX-4T-1 (GST-del1 to GST-del5) were obtained by cloning corresponding PCR fragments into the EcoRI/Sall-opened vector. The plant expression plasmid, pCypRS64-GFP, which expresses CypRS64 fused to the green fluorescent protein (GFP), was obtained by cloning a Sall/Smal CypRS64 PCR product into pBD-GFP (27) (obtained with Sall and SbfI (filled-in with Klenow). Five CypRS64 deletion mutants fused to GFP were obtained by cloning the corresponding PCR products as SalI/BglII (del1, del2, and del3) or Sall/BamHI (del4 and del5) into Sall/BamHI-opened pDEDH-GFP. Plasmids pSCL28-RFP, pSCL30-RFP, pSRP30-RFP, pSRP34-RFP, and pSLS33-RFP, which express red fluorescent protein (RFP, dsRED1, Clontech) fusions, and plant expression plasmids p70K-GFP and p70K-RFP, which express U1–70K tagged to GFP and RFP, have been described (28). Plasmids p70K-HA and p35K-HA, which express U1–70K and U1–35K fused to HA tag, and plasmids p35K-GFP and p35K-RFP, which express U1–35K fused to GFP and RFP will be described elsewhere.

**Yeast Two-hybrid Screening**—The two-hybrid screening was performed as described previously (26), and manipulation of the yeast strains was carried out according to the manufacturer’s instructions (Clontech).

**Protoplast Isolation, Transient Transformation, and Localization Studies**—Nicotiana tabacum leaf protoplasts were prepared and transiently transformed by the polyethylene glycol method as described in Refs. 29). Preparation and transient transformation of Arabidopsis cell suspension protoplasts was done as described in Meskiene et al. (30). Localization of GFP or RFP fusion proteins was analyzed in living cells 24 h after transformation on a Zeiss Axioplan and on a Leica TCS Confocal Scanning Microscope (Leica, Heidelberg).

**Overexpression of GST Fusion Proteins, Yeast, and Plant Protein Extracts Preparation and Pull-down Assay**—The GST-CypRS64, GST-del1, GST-del2, GST-del3, GST-del4, and GST-del5 were expressed in E. coli strain BL21 Codon plus-RIL (Stratagene). Overnight cultures grown at 37 °C in LB, in the presence of 100 µg/ml ampicillin and 40 µg/ml chloramphenicol, were diluted 100 times and further grown at 37 °C. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 2 h at 28 °C. Purification of fusion proteins on glutathione-Sepharose 4B beads (Amersham Biosciences), expression of HA-tagged proteins in yeast, extraction of proteins from yeast cells, and pull-down assay were described earlier (26). Plant protoplasts transformed with plasmids expressing HA-tagged proteins were collected 24 h later (15 min, 70 × g), frozen in liquid nitrogen, and resuspended in 300 µl (per 2 million protoplasts) of extraction buffer (PEB400; 50 mM HEPES-KOH, pH 7.9, 400 mM KC1, 2.5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Triton X-100), supplemented with EDTA-free protease inhibitor mixture (Roche Applied Science). Suspension was sonicated three times for 6 s, RNase A (final concentration of 0.45 µg/ml) and 100 µl of PEB400 were added, and the suspension was incubated on ice for 20 min with occasional mixing. After centrifugation in an Eppendorf centrifuge (15 min, maximum speed, 4 °C) concentration of KCl in the supernatant was adjusted to 200 mM (PEB200) with KC1-free PEB. Protein extracts were mixed with glutathione-Sepharose 4B beads coated with recombinant proteins in PEB200 or with beads alone, and incubated on a rotary shaker for 2 h at 4 °C. After four washings with PEB200, the beads were resuspended in 50 µl of loading buffer, and 25 µl were analyzed by SDS-PAGE and Western blotting.

**In Vitro Dephosphorylation of Transiently Expressed Proteins**—Protein extracts, prepared from transformed protoplasts as described above, were split into equal aliquots, and an indicated amount of calf intestinal phosphatase (CIP; New England Biolabs) was added. Extracts were incubated for 1 h at room temperature, and phosphatase was then inhibited by the addition of 3 µl of each phosphatase inhibitor mixture 1 and 2 (Sigma). At this point aliquots were taken for analysis of input extracts by Western blotting. Finally, glutathione-Sepharose 4B beads coated with GST-CypRS64 were added, and pull-downs were performed as described above.

**Immunoprecipitation**—Protein extracts, prepared from transformed protoplasts as described above, were incubated with 6 µg of anti-GFP monoclonal antibody (Roche Applied Science) for 1 h on ice and then mixed with protein-A-Sepharose and incubated additional 3 h at 4 °C. After three washings with 1 ml of PEB150 and one washing with 1 ml PEB400, the beads were resuspended in 50 µl of SDS-PAGE loading buffer, and 30 µl was analyzed by SDS-PAGE and Western blotting. Control immunoprecipitations were performed with protein-A-Sepharose beads alone. Protein extracts prepared from 2 million of the transformed protoplasts were used for one immunoprecipitation.

**SDS-PAGE and Western Blotting**—Proteins were run on 12% SDS-PAGE transferred onto a polyvinyldifluoride membrane (Millipore), and analyzed by Western blotting using standard procedure. Blots were developed by using an enhanced chemiluminescence system (Amersham Biosciences). Antibodies were diluted as follows: rat anti-HA monoclonal antibody 3F10 (Roche Applied Science), 1:1,000; rabbit anti-βt-IgG horseradish peroxidase-conjugated (Sigma), 1:10,000; mouse anti-monoclonal anti-GFP (Roche Applied Science) 1:1,000; and goat anti-mouse IgG horseradish peroxidase-conjugated (Bio-Rad) 1:10,000.

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a C. Forstner, S. Lopato, and A. Barta, unpublished data.

b Z. J. Lorković, R. Lehner, and A. Barta, manuscript in preparation.
FIG. 1. Sequence analysis of Arabidopsis CypRS64 and CypRS92. A, protein sequence of CypRS64. The PPIase domain is printed in blue letters, the SR/RS and SP dipeptides in red letters, and KRS domain on a yellow background. The arrowheads denote the starts of the three clones isolated in yeast two-hybrid screens. B, schematic drawings of the domain organization of CypRS64 and CypRS92. PPIase, peptidyl-prolyl cis-trans isomerase domain; KRS, region enriched in lysine, arginine, and serine residues; RS/SP, domain containing arginine/serine and serine/proline dipeptides. C, sequence alignment of the PPIase domains of CypRS64, CypRS92, and human SR domain-containing cyclophilins, SRCyp and NK-TR1, and Drosophila Moca-cyp. Amino acids identical or similar in 50% of the sequences are shaded on black and gray background, respectively.

RESULTS

Cloning and Sequence Analysis of Arabidopsis Cyclophilins, CypRS64, and CypRS92—Recently, a new group of SR-rich splicing factors, called SCL proteins has been described in Arabidopsis (26). To get more insight into their function we have performed yeast two-hybrid screenings with each member of this group of proteins. In addition to known Arabidopsis splicing factors, screens with SCL28 and SCL30 resulted in isolation of two partial cDNA clones encoding a protein containing numerous serine-arginine/serine-proline (SR/SP) dipeptides (hereafter RS/SP domain). A yeast two-hybrid screen with SRp30, an Arabidopsis homolog of the human SF2/ASF (31), cloning of the full-length cDNA revealed that it encodes a protein of 570 amino acids consisting of an N-terminal PPIase domain, followed by a region highly enriched in lysines, arginines, and serines (hereafter, KRS domain), and a C-terminal half of the protein enriched in SR/SP-dipeptides (Fig. 1, A and B). We named this protein CypRS64 (cyclophilin-containing RS domain of 64 kDa). In addition to the full-length cDNA (32; this work), an alternatively spliced form encoding a putative protein lacking RS/SP domain has been identified in the Arabidopsis expressed sequence tag data base. The Arabidopsis genome encodes an additional protein with the same domain organization as CypRS64 (Fig. 1B; gene code a4tg24240), and cloning of the full-length cDNA revealed that it encodes a protein of 837 amino acids (Supplemental Fig. S1) termed CypRS92, accordingly. CypRS64 and CypRS92 show high sequence similarity in the PPIase domain (58% identity and 72% similarity; Fig. 1C). The rest of the sequence is similar only in terms of being highly charged, enriched in serine residues, and having many SR/SP-dipeptides.

Data base searches with CypRS64 and CypRS92 revealed high sequence similarity with the human protein SRCyp (18, 19), its rat ortholog matrinCYP (20), and Drosophila Moca-cyp, which was isolated as an interacting partner of the transcriptional regulator p300/CPB (21). Sequence similarity between metazoan and Arabidopsis proteins is particularly high in the PPIase domain, with 52% identity and 64% similarity (Fig. 1C).

CypRS64 Interacts with Arabidopsis SR Proteins and with the snRNP-Specific Proteins U1–70K and U11–35K—Because all CypRS64 clones isolated in yeast two-hybrid screenings with SCL28, SCL30, and SRp30 proteins encoded only the RS/SP domain of the protein (Fig. 1A), we tested whether the full-length CypRS64 is also capable of interacting with SR proteins in a yeast two-hybrid assay. Co-expression of GAL4-CypRS64 DNA-binding domain fusion with SR proteins fused to GAL4 activation domain resulted in activation of both, His and β-galactosidase reporter genes (Fig. 2A). This confirmed the original two-hybrid interactions and showed that the full-length CypRS64 interacts with SCL28, SCL30, and SRp30. In a direct two-hybrid assay, we found that CypRS64 interacts with SRp34/SR1 (Fig. 2A), another Arabidopsis homolog of human SF2/ASF (31), and with SR protein-specific kinase SRPK4 (data not shown). An additional yeast two-hybrid screen with CypRS92 as a bait resulted in isolation of two partial cDNA clones encoding the C-terminal halves of the Arabidopsis U1 snRNP-specific protein U1–70K (33) and U11 snRNP-specific protein U11–35K (34); the same clones were found to interact with CypRS64 (Fig. 2B).

To test whether CypRS92 is also capable of interacting with Arabidopsis SR proteins in vivo, full-length cDNA encoding CypRS92 was cloned into a yeast two-hybrid vector fused to the GAL4 DNA-binding domain. The resulting plasmid was co-transformed into a yeast reporter strain with constructs expressing SCL28, SCL30, SRp34/SR1, U1–70K, and U11–35K fused to the GAL4 activation domain. All combinations resulted in efficient growth on medium lacking histidine, and in activation of the β-galactosidase reporter gene, indicating that all tested proteins interact with CypRS92 (Fig. 2, A and B).

To confirm the two-hybrid results, interactions between the proteins were also studied by in vitro pull-down assays. CypRS64 was overexpressed in Escherichia coli as GST fusion, bound onto glutathione-Sepharose beads, and incubated with protein extracts from yeast cells expressing HA-tagged SCL28, SCL30, SRp30, and SRp34/SR1. Western analysis of proteins retained on the beads with anti-HA antibody revealed that CypRS64 interacts with SCL28, SCL30, SRp30, and SRp34/SR1 (Fig. 2C, lanes 3 and 6). Pull-down experiments with GST or beads alone did not show any interactions with the proteins studied (Fig. 2C, lanes 2 and 5 and data not shown). Interactions between CypRS64 and U1–70K and U11–35K were also analyzed by in vitro pull-down assays by using, full-length HA-tagged U1–70K and U11–35K proteins expressed in plant
protoplasts. As shown in Fig. 2D (lanes 3 and 6), both proteins interacted efficiently with CypRS64, thereby confirming the yeast two-hybrid results. Because we were unable to express CypRS92 in E. coli, interactions of CypRS92 with SCL28, SCL30, SRp30, SRp34/SR1, U1–70K, and U11–35K were not analyzed by pull-down assay. Based on the two-hybrid and pull-down results we conclude that CypRS64 and CypRS92 interact with SR proteins, and with the snRNP-specific proteins U1–70K and U11–35K from Arabidopsis, suggesting their involvement in pre-mRNA processing.

In HeLa nuclear extracts U1–70K protein interacts with SR proteins, and this interaction is important for recruitment of U1 snRNP to the 5' splice site (reviewed in Refs. 3–5, 10). Therefore, we asked whether U1–70K and SRp34/SR1 interact in plant cells too. Immunoprecipitations with protein extracts prepared from tobacco protoplasts co-transformed with plasmids expressing U1–70K fused to HA-tag and SRp34/SR1 fused to GFP revealed efficient co-precipitation of the two proteins (Fig. 3, lanes 6). Because U11–35K protein was proposed to function as an analog of U1–70K in splicing of minor introns (34), we also found efficient co-precipitation of U11–35K and SRp34/SR1 (Fig. 3, lanes 4). This, together with binding of CypRS64 to SRp34/SR1, U1–70K, and U11–35K, indicates that interactions of U1–70K and U11–35K with SR proteins could be regulated by CypRS64 (see “Discussion”).

CypRS64 Is a Nuclear Protein—As CypRS64 interacts with SR proteins and snRNP-specific proteins, we were interested in its subcellular localization. To address this question we used the GFP-tagging approach. In transiently transformed tobacco leaf (Fig. 4, A and B, left panel) or Arabidopsis cell suspension protoplasts (Fig. 4B, two right panels), the CypRS64-GFP fusion was found in the nucleus in a diffuse pattern throughout the nucleoplasm, but excluded from nucleoli (Fig. 4B). Additionally, in 95% of transformed cells, fluorescence was found concentrated in one to six round dots, resembling Cajal bodies (CB, Fig. 4B). However, co-localization studies with U2B*, a protein marker for CBs in plant cells (28, 35, 36), and Nop10, a protein component of the small nucleolar ribonucleoprotein particles, which localize in CBs and nucleolus, revealed that these dots are not CBs (Fig. 4B, C and D, data not shown).

RS/SP and KRS Domains of CypRS64 Are Required for Interaction with SR and snRNP-Specific Proteins and For Correct Nuclear Localization of CypRS64—CypRS64 consists of three distinct structural domains (Fig. 1B). Because all CypRS64 two-hybrid positive clones encoded the RS/SP domain, we asked whether this is the only domain involved in protein-protein interaction. To this end, five deletion mutants of CypRS64 fused to GST were constructed and expressed in E. coli (Fig. 5, A and B). Pull-down experiments revealed that SCL28, SCL30, SRp30, and SRp34/SR1 require both the RS/SP and KRS domains for efficient binding, as equally efficient binding was observed with the full-length CypRS64 and del4 (Fig. 5C, compare lanes 3 and 7). In addition, each of these two domains alone (del3 and del5) is able to bind SR proteins with comparable efficiencies indicating that the overall strength of binding is the sum provided by these two domains (compare
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CypRS64 with U2B

Arrows indicate nuclear bodies.

lower panel

with anti-GFP antibody (upper panel)

noprecipitates obtained with anti-GFP antibody. Membrane was first incubated with protein-A-Sepharose beads only. Molecular mass standards are indicated on the left.

FIG. 3. Arabidopsis U1–70K and U11–35K proteins interact with SR protein SRp34/SR1 in vivo. Tobacco protoplasts were co-transformed with plasmids expressing SRp34/SR1 fused to GFP and U1–70K and U11–35K fused to HA tag. Protein extracts were prepared and used for immunoprecipitation with anti-GFP monoclonal antibody as described under “Experimental Procedures.” Lanes 1 and 2 were loaded with protein extracts (input). Lanes 3 and 5, protein extracts incubated with protein-A-Sepharose beads only. Lanes 4 and 6, immunoprecipitates obtained with anti-GFP antibody. Membrane was first probed with anti-HA-antibody (upper panel) and then after stripping with anti-GFP antibody (lower panel). Asterisks indicate immunoglobulin heavy chains.

lanes 6 and 8 with lanes 3 and 7). Simultaneous deletion of RS/SP and KRS domains (del2) abolished binding to all SR proteins (Fig. 5C, lanes 5), although weak binding to SRp30 and SRp34/SR1 was detected in some experiments (data not shown), indicating that the PPIase domain alone does not contribute significantly to protein-protein interaction with SR proteins. This is further supported by results obtained with del1 (Fig. 5C, lanes 4), which bound all SR proteins with approximately the same efficiency as del3 and del5. This, together with the yeast two-hybrid data, demonstrates that the protein-protein interactions of CypRS64 with SR proteins are mediated by the RS/SP and KRS domains. Similar results have been obtained with the U1–70K and U11–35K proteins, although binding to both del3 and del4 was weaker compared with SR proteins (Fig. 5C).

We also determined the sequence requirements for localization of CypRS64 in the nucleus by constructing five deletion mutants depicted in Fig. 5A fused to GFP. Confocal microscopy analyses of transformed protoplasts (Fig. 5D) revealed that the PPIase domain alone is not able to efficiently target the GFP fusion protein to the nucleus (del2; shown is the whole protoplast) and that it is not required for localization of the protein in “nuclear bodies” as the del4 mutant lacking PPIase domain was found, like the full-length CypRS64, diffusely distributed in the nucleoplasm (Fig. 5D, lower del4 image) and in nuclear bodies (Fig. 5D, arrows in upper GFP image of del4). Deletion of the PPIase and KRS domains (del5) resulted in localization of the fusion protein in a diffuse/speckled pattern in the nucleoplasm. Deletion mutant lacking RS/SP domain (del1) showed diffuse nucleoplasmic and nucleolar staining (Fig. 5D, broken arrows in del1). Some cells also showed localization in nuclear bodies (arrow in upper del1 GFP image). Expression of the KRS domain alone (del3) resulted in a diffuse nuclear localization of the fusion protein, which was, like del1, also found in nucleoli (broken arrows). Finally, deletion of both RS/SP and KRS domains (del2) resulted in cytoplasmic and nuclear (arrowhead) localization of the fusion protein. Nucleolar localization of del1 and del3 was confirmed by co-transformation with the nucleolar marker PRH75 fused to RFP (28). As shown in Fig. 5E both del1 and del3 localized to nucleoli as evident from the appearance of the yellow color (broken arrows) due to co-localization with PRH75, whereas del4 and del5, which were not seen in nucleoli did not show any co-localization with PRH75. In summary, localization of CypRS64 in nuclear bodies requires both RS/SP and KRS domains, although each of these two domains alone is sufficient to efficiently target the GFP fusion protein to the nucleus.

CypRS64 re-localizes from Nuclear Bodies into Speckles upon Co-expression With Its Interacting Partners—In accordance with their function in pre-mRNA splicing, SR proteins are known as nuclear proteins, localized in speckles (37), and this is true for plant SR proteins as well (Fig. 6, upper row (28)). The different subnuclear localization of CypRS64 and its interacting partners raised the question of how they interact in vivo in plant cells. To address this issue tobacco protoplasts were co-transformed with plasmids expressing CypRS64 fused to GFP and its interacting partners fused to RFP. Co-transformation of CypRS64 with SCL28, SCL30, SRp30, SRp34/SR1, U1–70K, and U11–35K showed co-localization of GFP and RFP signals (Fig. 6) and, interestingly, resulted in re-localization of CypRS64 from nuclear bodies into speckles (Fig. 6). In some cells, however, prominent nuclear bodies have been observed in which only CypRS64 was found (arrows in SRp30 and U11–35K images in Fig. 6, and data not shown). This was particularly evident in cells co-transformed with U11–35K. Co-expression of CypRS64 and RSZ33 (26), an Arabidopsis SR protein, which was not found to interact with CypRS64 did not result in re-localization of either CypRS64 or RSZ33 (Fig. 6). In addition, a nucleolar RNA helicase PRH75 (28, 38) also did not re-localize upon co-expression with CypRS64 (Fig. 6). Taken to-
gether, our co-localization studies indicate that re-localization of CypRS64 upon co-expression with its interacting partners is due to a functional interaction in vivo and not due to changes of nuclear or chromatin organization. Consistent with this are our observations that only those GFP-tagged deletion mutants of CypRS64 containing RS/SP domain co-localized with its interacting partners (data not shown). This is in line with the original two-hybrid data and in vitro interaction data with CypRS64 deletion mutants (Fig. 5C), thus confirming that the interaction between the proteins is mainly mediated by the RS/SP domain of CypRS64.

Phosphorylation Status of SRp34/SR1 Influences Its Interaction with CypRS64—SR proteins are phosphorylated in their RS domains, and the phosphorylation status of SR proteins influences their interaction with other splicing factors, distribution within the nucleus and recruitment to transcription initiation sites (reviewed in Refs. 39–41). Therefore, we asked whether phosphorylation of SR proteins influences their interaction with CypRS64. To address this question, protein extracts prepared from plant protoplasts expressing HA-tagged SR proteins were dephosphorylated with CIP for 1 h at room temperature and then used for pull-down assay with GST-
CypRS64. Control extracts were kept at room temperature without phosphatase treatment. Fig. 7A demonstrates that SRp34/SR1 and SCL30 are strongly phosphorylated when transiently expressed in plant protoplasts (compare input lanes −CIP and +CIP). In contrast, U11−35K protein (Fig. 7A) and U1−70K (data not shown) did not change their mobility on SDS-PAGE upon phosphatase treatment. Pull-down experiments revealed that dephosphorylation of SRp34/SR1 significantly reduced binding to GST-CypRS64 (Fig. 7A, compare lanes 4 and 5), whereas SCL30 and U11−35K (Fig. 7A, compare lanes 4 and 5) as well as SCL28 and U1−70K (data not shown) did not show this phosphorylation dependence. In addition, Western analysis of supernatants after incubation with GST-CypRS64 revealed that phosphorylated SRp34/SR1 is quantitatively depleted from the extract, whereas the dephosphorylated form is readily detectable (Fig. 7B, compare lanes 3 in −CIP and +CIP panels). To substantiate these data we performed dephosphorylation of SRp34/SR1 and SCL30 with increasing amounts of CIP. Pull-downs with corresponding extracts clearly show that increasing dephosphorylation of SRp34/SR1 (Fig. 7C, upper two panels) but not of SCL30 (Fig. 7C, lower two panels) strongly reduces its binding to GST-CypRS64, thereby confirming that phosphorylation of SRp34/SR1 is required for efficient binding to CypRS64.

**DISCUSSION**

CypRS64 and CypRS92 are large cyclophilins consisting of an N-terminal PPIase domain and a C-terminal domain enriched in SR/SP dipeptide repeats. This domain organization is similar to the recently described large nucleoplasmic cyclophilins from human (18, 19), rat (20), and Drosophila (21). We have demonstrated here that Arabidopsis RS domain-containing cyclophilins, CypRS64 and CypRS92, interact with a subset of Arabidopsis SR proteins and with U1 and U11 snRNPs-specific proteins U1−70K and U11−35K, respectively. CypRS64 is a nucleoplasmic protein; however, in transiently transformed protoplasts it accumulates in nuclear bodies of unknown origin, although cells showing speckled/diffuse nuclear localization pattern were also observed. In contrast, human SRCyp and its rat ortholog matrinCYP localized to nuclear speckles where they co-localized with SC35 and U1−70K proteins, respectively (19, 20). Interestingly, upon co-expression with its interacting partners, CypRS64 re-localized form nuclear bodies into speckles, subnuclear compartments in which SR proteins and snRNPs are found as well (reviewed in Refs. 37, 41, and 42), indicating functional interaction in vivo. Deletion analysis revealed that the RS/SP and KRS domains are mainly responsible for protein-protein interactions, for nuclear localization, and for accumulation of CypRS64 in nuclear bodies. Although we do not provide direct evidence for involvement of CypRS64 in splicing, our data clearly demonstrate interaction between CypRS64 and the splicing machinery, suggesting a function for CypRS64 in pre-mRNA splicing.

Splicing takes place in the spliceosome, a large ribonucleoprotein complex that assembles from five snRNPs and numerous additional protein factors. Spliceosome assembly is a highly ordered and dynamic process (reviewed in Refs. 6 and 7), and to date more than 200 spliceosomal proteins have been identified in humans (1, 2). Despite the fact that different spliceosomal complexes have been analyzed by mass spectrometry, human SRCyp has not been found in any of them (1, 2, 43–45). This might indicate that RS domain-containing cyclophilins only transiently associate with the assembling spliceosome.

Where in spliceosomal assembly could CypRS64 function? Based on our data on protein-protein interactions, revealing an interaction with SRp30 and SRp34/SR1, two Arabidopsis homologs of the human SF2/ASF, and with U1−70K protein, an appealing model would be that CypRS64 (and possibly CypRS92) are involved in early steps of spliceosomal assembly. In human cells, SF2/ASF binds to exonic splicing enhancers and interacts with U1−70K protein (10). This interaction is important for the initial association of U1 snRNP with the 5’ splice site. Consistent with a strong conservation of the splicing machinery between plants and metazoans (22; reviewed in Refs. 24 and 25), we have demonstrated here interactions between Arabidopsis U1−70K, as well as U11−35K, with SRp34/SR1 (Fig. 3). As splicing of minor, AT-AC introns also requires SR proteins (11), and our observation that CypRS64 interacts with U11 snRNP-specific protein U11−35K is consistent with this idea.

Phosphorylation of SR proteins is considered as an important means of regulation of SR protein function, and both hypo- and hyper-phosphorylation of SR proteins can affect their ability to interact with splicing complexes (Refs. 46 and 47; reviewed in Refs. 39–41). It is conceivable that CypRS64 is involved in regulation of SR protein phosphorylation/dephosphorylation by changing the accessibility of phosphorylation sites in their RS domains. Interestingly, phosphorylation of ASF/SF2 in the early spliceosomal complex is necessary for efficient interaction with U1−70K, but formation of catalytically active spliceosome requires its dephosphorylation (Ref. 48; reviewed in Refs. 39–
Thus, phosphorylation/dephosphorylation of SR proteins appears to accompany, and likely to regulate, the splicing cycle (Refs. 48 and 49; reviewed in Refs. 39–41). Two groups of kinases, Clk and SRPK, are being largely responsible for SR protein phosphorylation (reviewed in Refs. 39–42), and at least three different phosphatase activities have been reported to be required for splicing (48, 50–52). It can be speculated that some of these enzymes require specific conformation of the substrate, and a role for cyclophilin in this process can easily be envisaged. The highly repetitive sequence composition and the presence of many prolines in RS domains of SR proteins (particularly in Arabidopsis) indicate that they are rather unstructured. Having this in mind, peptidyl-prolyl cis-trans isomerase activity of CypRS64 could influence the conformation of RS domains by putting them into or keeping them in a more accessible conformation for protein-protein interactions or for phosphorylation/dephosphorylation. Interestingly, CypRS64 interacts with SRp34/SR1 in a phosphorylation-dependent manner suggesting that it might restructure SRp34/SR1 for dephosphorylation, or alternatively it may support binding of phosphorylated SRp34/SR1 to other splicing factors (e.g. U1–70K and U11–35K). In that respect, it is noteworthy that CypRS64 interacts also with SRPK kinases, and is a good SRPK substrate. Thus, phosphorylation of CypRS64 by SR protein-specific kinase could be a means of regulating its own activity as well. More detailed studies are required to get a coherent picture as to the regulation of CypRS64 function by phosphorylation, as well as on the requirements of SR protein phosphorylation for the interaction with CypRS64. Nevertheless, our data on protein-protein interactions and localization studies strongly suggest a role for RS domain-containing cyclophilins in spliceosomal dynamics.

Of note is that inhibition studies with the PPIase inhibitor cyclosporin A did not provide strong evidence for a requirement of this activity in pre-mRNA splicing in vitro (17). However, in vivo studies revealed that PPIase activity is required for splicing (17). The role of cyclophilins in pre-mRNA splicing in vitro, which takes at least 1 h may be masked by the relatively fast spontaneous isomerization of the prolyl bond. In contrast, splicing in vivo is very fast, thus supporting the requirement of PPIase activity for rapid conformational changes or dynamic protein-protein interactions occurring during the splicing cycle. Thus, subtle and rapid kinetic changes introduced by the PPIase activities in vivo may not be detected in a rather inefficient splicing reaction in vitro. Interestingly, proteomic analysis of purified spliceosomes or spliceosomal complexes revealed at least five cyclophilin candidates to be involved in splicing indicating importance of this class of proteins in splicing (1, 2, 43–45).

Although our data strongly suggest an involvement of RS domain-containing cyclophilins in splicing, it is possible that they are involved in other processes in the nucleus as well. For example, Drosophila and human RS domain-containing cyclophilins Moca-cyp and SRCyp have been found in a complex with the transcriptional regulator p300/CBP and the C-terminal domain of the RNA polymerase II, respectively (19, 21). This might indicate their involvement in transcriptional regulation of p300/CBP-responsive genes (21) or linking RNA polymerase II with the pre-mRNA processing (19). So far, we do not have any evidence for the interaction of RS domain-containing cyclophilins from Arabidopsis with the C-terminal domain of RNA polymerase II or with other nuclear proteins except those involved in splicing.

4 Z. J. Lorković and A. Barta, unpublished data.
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Interactions of Arabidopsis RS Domain Containing Cyclophilins with SR Proteins and U1 and U11 Small Nuclear Ribonucleoprotein-specific Proteins Suggest Their Involvement in Pre-mRNA Splicing

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