Interactions between two fission yeast serine/arginine-rich proteins and their modulation by phosphorylation

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The unexpected low number of genes in the human genome has triggered increasing attention to alternative pre-mRNA splicing, and serine/arginine-rich (SR) proteins have been correlated with the complex alternative splicing that is a characteristic of metazoans. SR proteins interact with RNA and splicing protein factors, and they also undergo reversible phosphorylation, thereby regulating constitutive and alternative splicing in mammals and *Drosophila*. However, it is not clear whether the features of SR proteins and alternative splicing are present in simple and genetically tractable organisms, such as yeasts. In the present study, we show that the SR-like proteins Srp1 and Srp2, found in the fission yeast *Schizosaccharomyces pombe*, interact with each other and the interaction is modulated by protein phosphorylation. By using Srp1 as bait in a yeast two-hybrid

analysis, we specifically isolated Srp2 from a random screen. This Srp interaction was confirmed by a glutathione-S-transferase pull-down assay. We also found that the Srp1–Srp2 complex was phosphorylated at a reduced efficiency by a fission yeast SR-specific kinase, Dis1-suppression kinase (Dsk1). Conversely, Dsk1-mediated phosphorylation inhibited the formation of the Srp complex. These findings offer the first example in fission yeast for interactions between SR-related proteins and the modulation of the interactions by specific protein phosphorylation, suggesting that a mammalian-like SR protein function may exist in fission yeast.

Key words: Dsk1 protein kinase, pre-mRNA splicing, Srp1 protein, Srp2 protein, two-hybrid analysis.

INTRODUCTION

Pre-mRNA splicing to remove introns from nuclear mRNA precursors is a crucial step in eukaryotic gene expression, and the modulation of pre-mRNA splicing is a widespread mechanism of gene control [1–3]. Alternative splicing to generate different protein isoforms from the same gene has gained central attention, particularly in the post-genomic era [4,5]. The unexpectedly low number of protein-coding genes, between 30 000–50 000, in the human genome [6,7], as compared with about 20 000 genes in a less sophisticated organism, the nematode *Caenorhabditis elegans* [8], has triggered increasing interest in pre-mRNA splicing. The genetic complexity required to progress from a primitive nematode to human and other mammals cannot be merely accomplished by the relatively small changes in gene numbers, and thus must rely largely on alternative splicing [4].

The concept of alternative splicing has been known for more than two decades, but the control mechanism has been slow to emerge. Alternative splicing as a control mechanism to select splice sites has been mainly studied in mammalian systems [9]. Perhaps the most important proteins that contribute to the splice-site selection through recognition of splicing enhancers are a family of RNA-binding proteins rich in serine and arginine residues [10,11]. These serine/arginine-rich (SR) proteins are evolutionarily conserved and have characteristic structural features with an N-terminal RNA-binding domain (RBD) and C-terminal arginine/serine-rich domain (RS domain) [12–15]. In

addition, the SR proteins are phosphorylated predominately on serine residues in the RS domain [14,16,17], presumably by SR protein-specific kinases, including the SR protein kinases (SRPKs) [17,18] and the Cdc2/Cdc28-like kinases (CLK/STY) [16]. The phosphorylation affects the cellular localization and the protein–RNA interactions of the SR proteins [19].

Although the studies in mammalian systems have revealed the role of SR proteins in alternative splicing, it remains largely unclear how SR proteins function, particularly *in vivo*. It is therefore important to find a genetically tractable and simple eukaryotic organism that has the features of SR proteins and alternative splicing similar to mammals. Since these features are not obvious in the budding yeast *Saccharomyces cerevisiae* [20,21], we have turned our attention to the fission yeast *Schizosaccharomyces pombe*. The genome sequence of *S. pombe* reveals that nearly 25% of its genes contain multiple introns [22,23], and some of them have been shown to undergo alternative splicing [24,25]. Moreover, SR-related proteins (Srps) and SR-protein-specific kinases exist in *S. pombe* [21,26]. Thus *S. pombe* has the potential to be a good model system for studying the function of SR proteins in splicing regulation.

Srp1 is the first protein isolated in fission yeast with typical features of mammalian SR proteins [27]. Srp1 contains one RBD at the N-terminus followed by an RS domain (Figure 1). The *srp1*⁺ gene is not essential for growth; however, overproduction of the RBD of Srp1 or the entire Srp1 protein with a mutant RS domain inhibits pre-mRNA splicing *in vivo*. Another SR-related

Abbreviations used: 3-AT, 3-aminotriazole; CLK/STY, Cdc2/Cdc28-like kinase; Dsk1, Dis1-suppression kinase; GAD, GAL4-activation domain; GBD, GAL4-DNA-binding domain; GST, glutathione S-transferase; Prp2, pre-RNA processing 2; RBD, RNA-binding domain; RS domain, arginine/serine-repeat domain; SF2/ASF, splicing factor 2/alternative splicing factor; SR protein, serine/arginine-rich protein; Srp, SR-related protein; SRPK, SR protein kinase; U2AF, U2 small nuclear ribonucleoprotein auxiliary factor.

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Figure 1 Structural domains of fission yeast Srp1 and Srp2 proteins

Each protein is divided into several regions in this representation, including RBD and RS domain. A glycine-rich domain (G) separates the RBD and RS domain in Srp1. The C-terminal region of Srp1 does not carry obvious similarity to motifs in the database. Srp2 contains two RBDs, RBD1 and RBD2, and SR/RS clusters (SR1 and SR2) flanking an arginine-rich domain (R). The highly conserved motifs, DARDA and SWQDLKD, which are found in RBD1 and RBD2 of all typical metazoan SR proteins are indicated.

protein in fission yeast, Srp2, harbours two RBDs, which are closely related to mammalian SR proteins SRp55, SRp75 and SRp40 [28]. Srp2 contains two short SR/RS repeat elements (SR1 and SR2) flanking an arginine-rich region (Figure 1), which is necessary for targeting the protein to the nucleus. The *srp2*⁺ gene is essential for cell viability, and its second RBD and the two SR elements are required for its function *in vivo*. SR protein-specific kinases are conserved in fission yeast [26,29]. Dis1-suppression kinase (Dsk1) protein, originally described as a mitotic regulator [30], displays high activity in phosphorylating *S. pombe* Srp1 and Srp2 proteins *in vitro* and is a functional homologue of human SRPK1 based on complementation assay *in vivo*. Another SR protein-specific kinase in fission yeast, Kic1, is an orthologue of mammalian CLK/STY according to sequence analysis and complementation assay *in vivo* (Z. Tang and R.-J. Lin, unpublished results). However, it is unclear whether Srp1 or Srp2 are genuine splicing factors and how Dsk1 or Kic1 regulates the function of these Srp proteins by phosphorylation.

To gain insights into the functions of Srp proteins and further explore *S. pombe* as a model system to study pre-mRNA splicing, we investigated protein-protein interactions of the *S. pombe* SR protein networks. By using Srp1 as bait in a yeast two-hybrid analysis [31], we specifically isolated Srp2 from a random screen. This Srp interaction was confirmed by a glutathione-S-transferase (GST) pull-down assay. We also found that the Srp1-Srp2 complex was phosphorylated at a reduced efficiency by Dsk1. Conversely, Dsk1-mediated phosphorylation inhibited the formation of the Srp complex. These findings offer the first example in fission yeast for interactions between SR-related proteins and the modulation of the interactions by specific protein phosphorylation, suggesting that a mammalian-like SR protein function may exist in fission yeast.

EXPERIMENTAL

Yeast two-hybrid screen

An *S. pombe* cDNA library from ClonTech was used, which was constructed by inserting the *S. pombe* cDNA sequences into the *GAL4*-activation domain (GAD) of the pGADGH plasmid. The plasmid carries the *LEU2* gene of *Saccharomyces cerevisiae*. A fragment containing the *S. pombe* *srp1*⁺ gene was subcloned downstream of and in-frame with the *GAL4*-DNA-binding domain (GBD) between *Bam*HI and *Sal*I sites in the vector pGBD-C1, which contains the *TRP1* gene [32]. *Saccharomyces cerevisiae* PJ69-4A (*MATa* *trp1*-901 *leu2*-3, 112 *ura3*-52 *his3*-200

gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ) was used as a host strain for transformation [32]. Sequential yeast transformation, selection and verification for positive interactions, and plasmid isolation were performed as directed by the ClonTech Matchmaker Library User Manual. Transformed cells were first plated on synthetic drop-out medium lacking leucine, tryptophan and adenine. A liquid assay for β -galactosidase activity was performed to examine a fraction of *ADE*⁺ colonies. Single colonies were re-streaked and subsequently grown on plates of synthetic drop-out medium without leucine, tryptophan and histidine. The *ADE*⁺*HIS*⁺ clones were re-screened for *HIS*⁺ expression in the presence of 2 mM, 5 mM, 7.5 mM or 10 mM 3-aminotriazole (3-AT). Colonies grown on medium without histidine and with 2 mM 3-AT were collected to establish the final pool of Srp1-interacting candidates. PCR primers for detecting *srp2* sequence in the candidate clones are one matching the upstream region of the pGADGH multiple cloning sites, GATGATGAAGATACCCACCAAACC, and one complementary to the *srp2*⁺ sequence, GAAGCGGGAGGGTACTTCGGGAAGG or GAGAACGACGGTATTCGTCTCGTCC.

Plasmids and protein production

Plasmids pET-28bGST, pET-28adsk1⁺, pET-28bGST-*prp2*⁺, pET-28asrp1⁺, pET-28bGST-*srp1*⁺, pET-28bsrp2⁺ and pET-28bGST-*srp2*⁺ have been described previously [26,29]. Recombinant proteins, GST-Srp1, Srp1, GST-Srp2, Srp2, GST-pre-RNA processing 2 (Prp2) and Dsk1, were expressed in the *Escherichia coli* strain BL21(DE3)pLysS, and the relative amounts of recombinant proteins in lysates were estimated as described previously [26,29].

GST pull-down assay

The detailed procedure has been described previously [26]. Briefly, two different bacterial lysates containing a GST-fusion protein and a non-GST-fusion protein were mixed at 23 °C for 30 min to allow complex formation. After the mixture was incubated with glutathione beads at 4 °C for 1 h, the bead-bound protein complexes were isolated by centrifugation and washing. The samples were processed for SDS/PAGE and Western-blot analysis. Immunoblotting in most experiments was performed as described previously [26,29]. When using 3C5 monoclonal antibody, 25 mM NaF and 1 mM NaVO₃ were present as phosphatase inhibitors to prevent dephosphorylation.

Kinase assay

Purified Dsk1 was incubated at 23 °C for 30 min with RS-domain-containing proteins in bacterial lysates or bound to glutathione beads in a total volume of 20–80 μ l in a kinase buffer (50 mM Tris/HCl, pH 7.4, 10 mM MgCl₂ and 1 mM dithiothreitol) with 50 μ M ATP and 0.1 μ Ci/ μ l [γ -³²P]ATP or with an ATP-regenerating system (10 mM creatine phosphate, 1 mM ATP and 0.1 mg/ml creatine phosphokinase) [26,29]. Protein phosphorylation was detected by autoradiography or by immunoblotting.

Phosphorylation/binding assay

The experiments in Figure 5 and Figure 6 (see below) combine the kinase assay *in vitro* with the GST pull-down assay. Bacterial lysate (20–40 μ l) containing a recombinant non-GST-fusion protein was incubated with 40 μ l of lysate containing a GST-fusion protein to form a complex at 23 °C for 15 min, and subsequently mixed with purified Dsk1 in kinase buffer in the presence of an ATP-regenerating system or [γ -³²P]ATP at 23 °C

for 30 min. To test the phosphorylation effect on complex formation, lysates containing a non-GST-fusion protein were first incubated with purified Dsk1, and then mixed with a lysate containing a GST-fusion protein. The Dsk1 was diluted 20 times in each reaction. The total volume for GST pull-down step was 100–150 μ l, and the procedure was carried out as described above.

Antibodies

To generate anti-Srp1 and anti-Srp2 polyclonal antibodies, two peptides corresponding to the N-terminal ends of Srp1 and Srp2 proteins, MSRRSLRTLYVTGFR and MSETRLVFGRIPPQA respectively, were conjugated to keyhole-limpet (*Diodora aspera*) haemocyanin. The conjugated peptides were injected into rabbits for immunization and the anti-sera from these rabbits were used to detect Srp1 and Srp2 proteins. Anti-(T7-Tag) monoclonal antibody was purchased from Novagen. Anti-GST polyclonal antibodies were from Santa Cruz Biotechnology. The monoclonal antibody mAb3C5 was obtained from mouse ascites.

RESULTS

Srp1 interacts with Srp2 in the yeast two-hybrid system

To investigate the interactions among elements of SR protein networks, we took a yeast two-hybrid approach to screen an *S. pombe* cDNA library using *srp1*⁺ gene as the bait. Putative interactions were identified based on the activation of both *ADE2*⁺ and *HIS3*⁺ reporter genes in the cells transformed with the cDNA library and the bait plasmid containing *srp1*⁺ gene. Transformants expressing the *ADE2*⁺ gene were initially selected and subsequently tested for *HIS3*⁺ gene expression in the presence of 2 mM 3-AT. As shown in Figure 2, cells transformed with only the *srp1*⁺ gene in either the bait or prey plasmid, pGBD-*srp1*⁺ or pGAD-*srp1*⁺, failed to grow in the absence of adenine (Figure 2, middle panel) or histidine (Figure 2, right panel). When the host strain was transformed with pGBD-*srp1*⁺ and an interacting plasmid, pGADGH-s86 or pGADGH-s184, the cells

Table 1 *S. pombe* cDNA library screen using Srp1 as bait

The yeast host strain, PJ69-4A, harbours three reporter genes, *GAL1-HIS3*, *GAL2-ADE2* and *GAL7-lacZ*. Srp1-interacting candidates were selected on the basis of the *ADE2*⁺ and *HIS3*⁺ gene expression. The *HIS3*⁺ gene expression was tested in the presence of different concentrations of 3-AT. In addition to be *ADE2*⁺, the colonies collected for the final pool were *HIS3*⁺ positive in 2 mM 3-AT. The identities of the candidates were determined by DNA sequencing or PCR using primers specific for detecting *srp2*⁺ sequence in the pGADGH plasmid.

Library size (independent clones)	No. of clones screened	No. of clones <i>ADE</i> ⁺ <i>HIS</i> ⁺ (2 mM 3-AT)	No. of clones analysed	No. of clones with <i>srp2</i> gene
5 × 10 ⁶	1.7 × 10 ⁶	137	90	89

grew without supplementation with adenine or histidine due to the activation of the reporter genes (Figure 2, middle and right panels). As summarized in Table 1, the screen reached more than one third of the independent clones present in the cDNA library, and the double selection scheme resulted in 137 *ADE2*⁺*HIS3*⁺ clones potentially encoding interacting factors.

Initially, eight cDNA clones from the pool were sequenced; seven clones carried *srp2*⁺-coding sequence and one contained a gene of a translation elongation factor (results not shown). Clone s86 contained the entire coding sequence of the *srp2* gene, whereas s184 represented the shortest *srp2* clone, missing about 150 nucleotides at the 5' end of the *srp2*⁺ gene. We next analysed 82 additional clones by PCR using primers from the internal coding region of the *srp2*⁺ gene. All 82 clones contained the *srp2* sequence (Table 1). The results rule out the possibility that the prevalence of the *srp2* sequence in the candidate pool was due to contamination of *srp2* DNA, since amplification of the *srp2* sequence was not observed in parallel PCR reactions of other screens with different bait. Also, it is unlikely that Srp2 interacts with the binding domain of Gal4 to activate the reporter genes, because no *srp2* clones appeared when other genes were used as the bait (results not shown). Further, Srp2 itself does not act as

Figure 2 Srp1 interacts with Srp2 in a yeast two-hybrid system

The *srp1*⁺ gene was subcloned downstream of the *GAL4* DNA-binding domain in vector pGBD-C1 and used as bait to screen yeast an *S. pombe* cDNA library. Strain PJ69-4A transformed with various bait and prey plasmids as indicated were streaked on to plates with (+) or without (–) histidine (His) or adenine (Ade) and incubated at 30 °C for 2–3 days. Note that both pGADGH-s86 and pGADGH-s184 contained a *srp2*⁺ coding sequence fused to the activation domain sequence of *GAL4*. An interaction between the Srp1 and the prey protein turned on the expression of the reporter genes, *ADE2*⁺ and *HIS3*⁺, which resulted in cell growth.

Figure 3 Srp1 and Srp2 proteins interact with each other in a GST pull-down assay

A bacterial lysate containing GST-Srp1 (lanes 4–12) or GST-Srp2 protein (lanes 16–24) was mixed with Srp1-containing lysate (lanes 7–9 and 22–24), Srp2-containing lysate (lanes 10–12 and 19–21), or no additional lysate (lanes 4–6 and 16–18) to allow complex formation at 23 °C for 30 min. Glutathione beads were then added to pull down bound proteins at 4 °C as described in the Experimental section. To distinguish non-specific binding, a lysate containing GST protein was used in place of a GST-fusion protein (lanes 1–3 and 13–15). The samples were immunoblotted with anti-(T7-Tag) monoclonal antibody. Alkaline phosphatase-conjugated goat anti-mouse IgG was used as the secondary antibody. The identity of the proteins is marked above the bands in mix and bound fractions of the samples with a number from 1 to 5 representing GST, Srp1, Srp2, GST-Srp1 and GST-Srp2 respectively, as indicated on the right side of the figure. Approx. 10% of the mixed lysates and unbound fraction from each sample were loaded on to the gel.

a transcription factor, since a number of *srp2* clones tested became *ADE2⁻HIS3⁻* after the bait plasmid pGBD-*srp1⁺* was eliminated. Therefore, the Srp1 dependence and the high frequency of the *srp2* gene in the selected clones provided molecular genetic evidence for a specific interaction between the Srp1 and Srp2 proteins. The result suggests that Srp1 may form a complex with Srp2 in *S. pombe* cells. Moreover, the isolation of clone s184 indicates that the N-terminal 51 amino acids of Srp2 protein, including the DARDA signature motif in RBD1 (Figure 1), are dispensable for the interaction.

Srp1 and Srp2 form a complex *in vitro*

To verify the binding of Srp1 to Srp2, we used a GST pull-down assay. Recombinant T7-epitope-tagged Srp1 and Srp2 proteins were produced in bacteria in two versions: one with and one without GST fusion. Lysates containing GST-fusion proteins were incubated with lysates containing non-GST-fusion proteins. Glutathione-agarose beads were added to bind the GST fusions and the interacting proteins. Portions of the mixed lysates, unbound and bound fractions from each sample were analysed by Western blotting using an anti-(T7-Tag) monoclonal antibody (Figure 3). Consistent with the two-hybrid results, Srp2 was co-precipitated with GST-Srp1 (Figure 3, lane 12). Similarly, in the reciprocal pair, Srp1 was brought down with GST-Srp2 (Figure 3, lane 24). In contrast, neither Srp1 (Figure 3, lane 9) nor Srp2 (Figure 3, lane 21) associated with its own GST-fusion protein. Specific protein bands in 'bound' fractions were recognized and distinguished from non-specific protein bands by comparison with the GST-Srp1 containing lysate (Figure 3, lanes 4–6) or GST-Srp2 containing lysate (Figure 3, lanes 16–18). Possibly, as a result of GST-Srp2 degradation, a protein band migrating near Srp1 was observed in all 'bound' fractions con-

taining GST-Srp2 (Figure 3, lanes 18, 21 and 24). Since the anti-(T7-Tag) monoclonal antibody could not distinguish Srp1 from GST-Srp2, we used Srp1-specific polyclonal antibodies to resolve the ambiguity. Srp1 was specifically and solely detected in the 'bound' fraction derived from the mixed lysates of Srp1 and GST-Srp2, but was not detected in samples lacking Srp1 (results not shown). As negative controls, a lysate containing GST protein was used to replace the GST-fusion protein to distinguish non-specific binding (Figure 3, lanes 1–3 and 13–15). Neither Srp1 (Figure 3, lane 3) nor Srp2 protein (Figure 3, lane 15) co-precipitated with the GST protein. Therefore, these results provide *in vitro* evidence for the association between Srp1 and Srp2.

We next tested whether Srp1 or Srp2 protein bound to other *S. pombe* RS-domain-containing proteins, such as Prp2. Prp2, also named spU2 small nuclear ribonucleoprotein auxiliary factor 59 (spU2AF59) due to its homology with the large subunit of human U2AF, is required for pre-mRNA splicing in fission yeast. In contrast with Srp1, Srp2 and typical mammalian SR proteins, Prp2 has an RS domain in the N-terminal region and an RBD in its C-terminal region [33]. In these experiments, a lysate containing GST-Prp2 protein was incubated with a lysate containing Srp1 or Srp2 protein. After the GST-fusion proteins were isolated by glutathione-agarose beads, samples were processed for SDS/PAGE, and subsequently analysed by Western blotting using the anti-(T7-Tag) monoclonal antibody (for detecting Srp1 and Srp2) and anti-GST polyclonal antibodies (for detecting GST-Prp2) (Figure 4). As displayed in Figure 4, neither Srp1 (lane 3) nor Srp2 (lane 6) co-precipitated with GST-Prp2. This is consistent with the observation that the *prp2* sequence was not found among the 90 Srp1-interacting clones in the two-hybrid screen (Table 1). The associations among these RS-domain-containing proteins *in vitro* are summarized in

Figure 4 Neither Srp1 nor Srp2 protein interacts with Prp2 protein in GST pull-down assays

A bacterial lysate containing GST–Prp2 was mixed with a lysate containing Srp1 (lanes 1–3) or Srp2 protein (lanes 4–6). The samples were processed as described in Figure 3. Anti-(T7-Tag) monoclonal antibody (for detecting Srp1 and Srp2) and anti-GST polyclonal antibodies (for detecting GST–Prp2) were used in immunoblotting. Numbers 1, 2 and 3 below the protein bands stand for Srp1, Srp2 and GST–Prp2 respectively, as indicated on the right side of the figure. Approx. 6% of the mixed lysates and unbound fraction from each sample were loaded on to the gel.

Table 2 Complex formation *in vitro* among various RS-domain-containing proteins

GST-fusion protein	Non-GST protein	
	Srp1	Srp2
GST	–	–
GST–Srp1	–	+
GST–Srp2	+	–
GST–Prp2	–	–

Table 2. These results indicate that Srp1 and Srp2 do not bind to Prp2 or to themselves, but they do specifically interact with each other.

Complex formation decreases Dsk1-mediated phosphorylation

Srp1 and Srp2 are individually phosphorylated by Dsk1 *in vitro*, and the phosphorylation generated the same phospho-epitope identified in mammalian SR proteins [26]. In the present study we tested whether Dsk1 could phosphorylate Srp1 and Srp2 in the complex. Srp1–Srp2 complexes formed in bacterial lysates were isolated on glutathione–agarose beads and incubated with or without purified Dsk1 in the presence of [γ - 32 P]ATP (Figure 5). As previously shown [26], GST–Srp1 and GST–Srp2 were individually phosphorylated, and the phosphorylation was Dsk1 dependent (Figure 5, lanes 2 and 6). The lower molecular-mass band observed in lane 2 was likely to be a degradation product of GST–Srp1. Similar amount of GST–Srp1 and GST–Srp2 were added to samples shown in lanes 2 and 4, and lanes 6 and 8, respectively (Figure 5). However, both proteins, GST–Srp1 and GST–Srp2, were phosphorylated by Dsk1 at lower levels when

complexed with their partner, as compared with phosphorylation of each protein alone (Figure 5, compare lanes 2 and 4, and lanes 6 and 8). The analysis of the PhosphorImager data indicated that the intensity of the GST–Srp1 band in lane 2 (Figure 5) was 2.4 times that of the GST–Srp1 band in lane 4, and the intensity of the GST–Srp2 band in lane 6 was 3.3 times that of the GST–Srp2 band in lane 8. Although the association between Srp1 and Srp2 did not completely sequester phosphorylation sites, nevertheless, Dsk1 phosphorylated each complexed protein less efficiently.

Dsk1-mediated phosphorylation decreases the gel mobility of Srp1 and Srp2 [26]. To confirm that the phosphorylation of Srp proteins by Dsk1 was affected by complex formation, we performed immunoblotting experiments to test whether the underphosphorylated status of the Srp proteins could be revealed by the lack of mobility changes on a gel. Bacterial lysates containing GST–Srp2 or GST–Srp1 were mixed with lysates containing Srp1 or Srp2 to form complexes. Purified Dsk1 was added and the mixture was incubated in the presence of an ATP-regenerating system. After the kinase reaction, the complexes were isolated by binding to glutathione–agarose beads and subjected to Western-blot analysis using anti-Srp1 or anti-Srp2 polyclonal antibodies (Figure 6A, lanes 1–3 and lanes 7–9). The gel mobility of the complexed Srp1 or Srp2 did not change upon incubating with Dsk1 (Figure 6A, compare lanes 7 and 9, and lanes 1 and 3). Gel-mobility changes were clearly detected when Srp1 or Srp2 was incubated with Dsk1 (Figure 6A, lanes 11 and 5, and also [26]). Apparently, after phosphorylation by Dsk1, the mobility of Srp2 (Figure 6A, lane 3) or Srp1 (lane 9) in the complex did not change as much as the mobility of the non-complexed protein (lanes 5 and 11 respectively). Thus, Srp1 and Srp2 were indeed phosphorylated at lower levels in the complex. The reduction of phosphorylation in the complex was not due to insufficient supply of Dsk1, since half of the amount of Dsk1 was sufficient to cause gel mobility shift of either Srp protein.

Dsk1-mediated phosphorylation inhibits interaction between Srp1 and Srp2

To examine the relationship between Srp interaction and Dsk1-mediated phosphorylation, we combined the *in vitro* kinase reaction with the GST pull-down assay. Lysates containing Srp1 or Srp2 were first incubated with purified Dsk1 in a kinase buffer and subsequently mixed with a lysate containing a GST-fusion partner for complex formation. The phosphorylation status of the Srp proteins was revealed by gel mobility shift, as analysed by Western blots using anti-Srp1 or anti-Srp2 polyclonal antibodies (Figure 6A, lanes 4–6 and lanes 10–12). Both Srp1 and Srp2 migrated more slowly after the kinase reaction (Figure 6A, lanes 11 and 5) than before the reaction (lanes 10 and 4), indicating that they were highly phosphorylated by Dsk1 [26]. Upon phosphorylation, much less Srp1 protein was pulled down with GST–Srp2 (lane 12), as compared with the amount without Dsk1-mediated phosphorylation (compare with Figure 3, lane 24). In addition, the Srp1 in the bound fraction contained mainly fast migrating or less phosphorylated forms (Figure 6A, lane 12). On the contrary, the Srp2 in the bound fraction appeared as a broad slow-migrating band (Figure 6A, lane 6), suggesting that phosphorylated Srp2 could still associate with GST–Srp1 quite efficiently.

We further tested the effect of Dsk1-mediated phosphorylation on complex formation by carrying out the kinase reaction in the presence of [γ - 32 P]ATP (Figure 6B), so that the relative amounts of phosphorylated Srp proteins in the bound and unbound

Figure 5 Dsk1-mediated phosphorylation of Srp proteins assayed by ^{32}P -labelling

Srp1, Srp2 and the complex were isolated by GST pull-down as described in Figure 3. The samples were incubated with (+) or without (–) purified Dsk1 in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ at 23 °C for 30 min. Samples were resolved by SDS/PAGE (10% gel) and visualized by autoradiography. The positions of Srp1 (lane 8), Srp2 (lane 4), GST–Srp1 (lanes 2 and 4) and GST–Srp2 (lanes 6 and 8) on the gel are indicated on the right. Truncated GST–Srp1 protein was observed as a lower molecular-mass band (lane 2) marked with an asterisk.

fractions could be analysed directly. ^{32}P -labelled Srp2 was detected in the bound fraction (Figure 6B, lane 3), although the binding efficiency was decreased compared with that of the unphosphorylated counterpart (compare with Figure 3, lane 12). Very little phosphorylated Srp1 was co-isolated with GST–Srp2 (Figure 6B, lane 6), indicating that Dsk1-mediated phosphorylation of Srp1 abrogated complex formation. Therefore the

interaction between Srp1 and Srp2 proteins is particularly inhibited by Dsk1-mediated phosphorylation of Srp1. The experiments were repeated several times and the results were reproducible.

DISCUSSION

We have shown that the *S. pombe* Srp1 and Srp2 proteins interact with each other in a yeast two-hybrid system and the N-terminal 51 amino acids residues of the Srp2 protein, including the DARDA signature motif in RBD1, are not required for this interaction. The two proteins form a complex *in vitro*, and both proteins in the complex can be phosphorylated by fission yeast Dsk1. However, the phosphorylation of both Srp proteins by Dsk1 is reduced in the complex. Conversely, Dsk1-mediated phosphorylation of Srp1 or Srp2 interferes with the interaction; particularly, phosphorylation of Srp1 by Dsk1 virtually disrupts its binding to Srp2. This is the first description of an interaction occurring between two SR-related proteins and the modulation of such interaction by specific protein phosphorylation in fission yeast. Further exploration of the system will potentially fill the gap in *in vivo* systems available for the study of splicing control.

The significance of the Srp interaction described here lies in the specificity of the binding between the two proteins. We employed a two-hybrid scheme to look for the interacting factors of Srp1 in a random screen. As a result, almost all of the interacting clones selected from the *S. pombe* cDNA library contained *srp2*⁺-coding sequences (Table 1), providing strong evidence for the specificity of the interaction. We did not find Dsk1 among the Srp1-interacting clones that we analysed, although Dsk1 forms a complex with Srp1 *in vitro* [26]. One explanation for this may be attributed to the temporal nature of their binding *in vivo*, since Dsk1 is released from the complex after the phosphorylation

Figure 6 Dsk1-mediated phosphorylation of Srp proteins and the effect of phosphorylation on Srp complex formation

(A) Gel-mobility shift assays using Western-blot analysis. Srp proteins were phosphorylated by Dsk1 either before (lanes 5 and 6, 11 and 12) or after (lanes 2 and 3, 8 and 9) Srp-complex formation. To phosphorylate before Srp-complex formation, lysates containing Srp1 (lanes 11–12) or Srp2 (lanes 5–6) were incubated with Dsk1 and ATP at 23 °C for 30 min, and subsequently mixed with a lysate containing GST–Srp2 or GST–Srp1 at 23 °C for 15 min for complex formation. To phosphorylate after Srp-complex formation, bacterial lysates containing GST–Srp1 or GST–Srp2 were incubated with lysates containing Srp2 (lanes 1–3) or Srp1 (lanes 7–9) at 23 °C for 15 min to allow complex formation. Purified Dsk1 and ATP were then added and incubated at 23 °C for 30 min. Following these manipulations, the reaction mixtures were incubated with glutathione–agarose beads and subjected to Western-blot analysis using anti-Srp1 (αSrp1) or anti-Srp2 (αSrp2) polyclonal antibodies. Lanes 1, 4, 7 and 10 were lysates (10% compared with the kinase-treated samples) without the kinase treatment and without GST pull-down. Approx. 30% of the unbound fraction from each sample were loaded on to the gel. (B) ^{32}P -labelling assay. Srp1 (lanes 4–6) or Srp2 (lanes 1–3) were phosphorylated by Dsk1 in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ prior to the Srp-complex formation and GST pull-down. The experiments were essentially carried out as described in (A). Similarly, approx. 10% of the mixed lysates and 30% of the unbound fraction from each sample were loaded on to the gel. Protein bands were visualized by phospho-imaging and autoradiography. The positions of the phosphorylated Srp1 and Srp2 proteins on the gel are indicated on the right- and left-hand sides respectively.

reaction *in vitro*. The specificity of the Srp interaction was further illustrated in the GST pull-down assay. Srp1 and Srp2 were bound together, but associations of either Srp1 or Srp2 with itself or with Prp2, a fission yeast RS-domain-containing protein, were not detected under the same condition (Figure 4). Consistent with the specificity in the interactions involving Srp1 and Srp2, specific protein–protein interactions among RS-domain proteins have been shown in mammalian systems, which play key roles in early steps of spliceosome assembly [10,11,34]. Therefore the specificity of the Srp interaction supports the notion that Srp1 and Srp2 are likely to function in pre-mRNA splicing [28,35] and that they associate with each other in their action.

The phosphorylation of Srp1 by Dsk1 exerted a stronger inhibition effect on the Srp-complex formation than that of Srp2 (Figure 6). It has been shown that phosphorylation of an SR protein can have various effects on its interactions with different RS-domain-containing proteins in mammalian cells [10,11,19] or in *Drosophila* [36,37]. For example, the phosphorylation status of splicing factor 2/alternative splicing factor (SF2/ASF) differentially affects its associations with several SR-related proteins *in vitro* [38]. Phosphorylation of SF2/ASF protein enhances its interaction with U1 snRNP-specific 70 kD protein *in vitro* [39], perhaps by strengthening the ionic interaction of RS domains through constituting a 'polar zipper' [40]. On the other hand, SF2/ASF phosphorylation decreases its binding to SRp40 and has no obvious influence on its association with U2AF35 [38].

The change in the binding property of the Srp proteins by Dsk1-mediated phosphorylation may affect the molecular composition of the splicing machinery. For instance, if the association of each Srp protein with the spliceosome and the formation of the Srp complex are mutually exclusive, Dsk1-mediated phosphorylation may allow an individual Srp to bind to the splicing machinery. Conversely, if Srp proteins are capable of interacting with both the spliceosome and with each other simultaneously, recruitment of Srp proteins into the spliceosome is then dependent on the phosphorylation status of each Srp.

It is also possible that Dsk1-mediated phosphorylation affects the cellular localization of Srp1 or Srp2. The two SR elements of Srp2 are necessary for targeting the protein into the nucleus [28], which is reminiscent of the role for the RS domain of mammalian SR proteins in nuclear import [41,42]. The phosphorylation of the RS elements may inactivate the nuclear localization signal via a masking mechanism [43]. For example, phosphorylation of the most C-terminal SR/RS dipeptide of budding yeast Npl3 by the Sky1 kinase is required for the nuclear import of Npl3 [44]. In addition, Sky1-mediated phosphorylation changes protein interactions and cellular localization of several mammalian SR proteins expressed in budding yeast [45]. Thus decrease in phosphorylation upon Srp1 binding may affect the nuclear import of Srp2. Interestingly, Dsk1 itself displays a cell-cycle-dependent pattern of cellular localization. It is localized mainly in cytoplasm during interphase and in the nucleus at mitosis [30]. Therefore Dsk1 in the cytoplasm may phosphorylate Srp proteins in interphase to regulate their nuclear import and splicing activity.

Our findings provide the first evidence for the interactions of *S. pombe* SR-related proteins and the regulation of the interactions by phosphorylation. The results support the view that phosphorylation of SR-related proteins may serve as a mechanism for pre-mRNA splicing regulation in *S. pombe*, particularly considering that *S. pombe* has many introns with degenerate consensus sequences [22,46–48] and the polypyrimidine tract downstream from the branch point has a similar function in splicing as it does in mammals [49]. Further studies using *S. pombe* are likely to provide insights into the mechanisms of splicing regulation fundamental to higher eukaryotes.

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