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## Fission Yeast Mitotic Regulator Dsk1 Is an SR Protein-specific Kinase\*

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Intricate interplay may exist between pre-mRNA splicing and the cell division cycle, and fission yeast Dsk1 appears to play a role in such a connection. Previous genetic analyses have implicated Dsk1 in the regulation of chromosome segregation at the metaphase/anaphase transition. Yet, its protein sequence suggests that Dsk1 may function as a kinase specific for SR proteins, a family of pre-mRNA splicing factors containing arginine-serine repeats. Using an *in vitro* system with purified components, we showed that Dsk1 phosphorylated human and yeast SR proteins with high specificity. The Dsk1-phosphorylated SF2/ASF protein was recognized strongly by a monoclonal antibody (mAb104) known to bind the *in vivo* phosphoepitope shared by SR proteins, indicating that the phosphorylation sites resided in the RS domain. Moreover, the fission yeast U2AF65 homolog, Prp2/Mis11 protein, was phosphorylated more efficiently by Dsk1 than by a human SR protein-specific kinase, SRPK1. Thus, these *in vitro* results suggest that Dsk1 is a fission yeast SR protein-specific kinase, and Prp2/Mis11 is likely an *in vivo* target for Dsk1. Together with previous genetic data, the studies support the notion that Dsk1 may play a role in coordinating pre-mRNA splicing and the cell division cycle.

Many cellular processes are regulated coordinately with the progression of the cell division cycle (e.g. Refs. 1 and 2), and RNA splicing is likely to be included in this regulation. For example, RNA splicing may be down-regulated in the cell when transcription is repressed during mitosis and up-regulated when RNA precursors are produced actively during cell growth (3). Indeed, evidence indicates that the pre-mRNA splicing apparatus is disassembled during mitosis and has to be reassembled when cells exit mitosis (4). Because protein phosphorylation is involved in the regulation of virtually every aspect of cellular processes (5, 6), we decided to look for protein kinases in fission yeast which are involved in regulating splicing during the cell cycle. The Dsk1 kinase in *Schizosaccharomyces pombe* is an excellent candidate because it has a role in cell cycle progression (7), and it is homologous to a human SR protein kinase (8). Compared with budding yeast, fission yeast (*S. pombe*) has the advantage of being more similar to higher eukaryotes especially with regard to the appearance of introns in protein-encoding genes (9–11).

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The *dsk1*<sup>+</sup> gene was originally identified as a multicopy suppressor of cold-sensitive *dis1* mutants (7). *dis1* mutants are defective in sister chromatid separation at the restrictive temperature, and mitosis never reaches completion in these mutants (12). The Dis1 protein is associated with microtubules and the spindle pole body and probably is phosphorylated by Cdc2 kinase (13). *dsk1*<sup>+</sup> gene is not essential for viability, probably because of a redundancy in its function in fission yeast, but overexpression of *dsk1*<sup>+</sup> results in a delay at the G<sub>2</sub>/M phase transition (7). *dsk1*<sup>+</sup> encodes a 61-kDa protein kinase, but its *in vivo* substrate has yet to be identified. Although *dis1* mutants are suppressed by increasing the expression level of *dsk1*<sup>+</sup> (7), the Dis1 protein is unlikely as a substrate for the Dsk1 kinase because *dsk1*<sup>+</sup> also suppresses a null allele of *dis1*.<sup>1</sup> Dsk1 itself becomes highly phosphorylated at mitosis, and the Dsk1 protein isolated from mitotic cells is more active in phosphorylating myelin basic protein (MBP)<sup>2</sup> *in vitro* (7). Interestingly, the localization of Dsk1 is also cell cycle-dependent; Dsk1 is localized in the cytoplasm during interphase, but it is found mostly in the nucleus at mitosis. Hence the Dsk1 protein may play a role in mitotic control by altering its cellular location and its target proteins.

The sequence similarity between Dsk1 and human SRPK1 (SR protein kinase 1) suggests that the *in vivo* substrates for Dsk1 may be proteins containing serine/arginine repeats. SRPK1 specifically phosphorylates a family of pre-mRNA splicing factors called SR proteins, at their arginine/serine-rich domain, the RS domain (8, 14). SR proteins are involved in constitutive splicing (15, 16) as well as being specific modulators in alternative splicing (17). *In vivo*, SR proteins are phosphorylated, predominantly on serine residues in the RS domain (18, 19). The phosphorylation of SR proteins apparently is not only important for the splicing reaction itself but also affects the location of the SR proteins within the nucleus. A cycle of phosphorylation-dephosphorylation of splicing factors is necessary for splicing to take place (20, 21). The stage-dependent sensitivity of pre-mRNA splicing to phosphatases and phosphatase inhibitors observed in mammalian nuclear extracts may simply reflect the dynamics in differential phosphorylation of SR proteins in one round of the splicing reaction. Recent evidence demonstrates that phosphorylation of SR proteins enhances protein-protein interactions while also inhibiting non-specific interactions with RNA (22). The phosphorylation cycle of splicing factors is reminiscent of the mutually antagonistic kinase and phosphatase systems operating cell cycle control (23, 24). Thus, the cellular localization and possibly the activity of SR proteins are regulated during the cell cycle by their phosphorylation status in a stage-specific manner (4).

<sup>1</sup> M. Yanagida, unpublished observations.

<sup>2</sup> The abbreviations used are: MBP, myelin basic protein; SRPK1, SR protein kinase 1; GST, glutathione S-transferase; mAb, monoclonal antibody.

Prp2 protein is the only SR-like protein identified so far which is required for pre-mRNA splicing in *S. pombe*. The *prp2*<sup>+</sup> gene encodes a homolog of the 65-kDa subunit of human splicing factor U2AF (25), and human U2AF65 protein is a good substrate for SRPK1 *in vitro* (8). A *prp2* mutant was identified initially as a temperature-sensitive mutant defective in pre-mRNA splicing (26). Interestingly, another *prp2* mutant allele, *mis11*-453, was isolated by a screen for mutants impaired in chromosome segregation with a high rate of minichromosome loss (27). Progression through G<sub>1</sub> and G<sub>2</sub> phases is blocked in *mis11* mutant cells which leads to reduced cell size. Thus, the Prp2/Mis11 protein is a very attractive candidate as a substrate for Dsk1.

The fission yeast Dsk1 protein is of particular interest because of its possible function in coordinating pre-mRNA splicing with the progression of the cell division cycle. It may provide a model system to unravel, at the molecular level, the mechanism for synchronous regulation between pre-mRNA splicing and the cell division cycle. To determine whether Dsk1 is a functional homolog of human SRPK1 in fission yeast, we purified recombinant Dsk1 and various SR proteins to characterize the kinase properties of Dsk1. Using a cell-free assay we established biochemically that Dsk1 is an SR protein-specific kinase in fission yeast. The results also provided the first evidence that the essential splicing factor in *S. pombe*, the Prp2/Mis11 protein, is likely an *in vivo* target for Dsk1 function. Our studies, combined with the previous genetic data, suggest that Dsk1 may be a dual functional protein involved in both pre-mRNA splicing and the cell division cycle.

#### EXPERIMENTAL PROCEDURES

**Plasmid Construction**—To construct pET-28a *dsk1*<sup>+</sup> encoding Dsk1 with a histidine tag at the NH<sub>2</sub> terminus, a fragment of 340 base pairs from the 5'-portion of *dsk1*<sup>+</sup> (7) was synthesized using two primers (5'-GCCAGCCATGGATCCATGGGAAGTG-3', including a *Bam*HI site, and 5'-GGCAGCTCGATCATATGCAAGCCAAAC-3', including the unique internal *Nde*I site) in a polymerase chain reaction (28). The polymerase chain reaction fragment was inserted into the PCR II vector (Invitrogen). The *Nde*I-*Eco*RV fragment of 1,400 base pairs from pDS113-6 (7), including the remainder of *dsk1*<sup>+</sup>, was added to regenerate the entire *dsk1*<sup>+</sup> coding sequence. The *Bam*HI/*Not*I fragment containing the constructed *dsk1*<sup>+</sup> was then inserted into pET-28a (Novagen) to generate pET-28a *dsk1*<sup>+</sup>. To construct pET-28b GST-*prp2*<sup>+</sup> encoding Prp2 fused at the COOH terminus of glutathione *S*-transferase (GST), the *Nco*I-*Nde*I fragment in pET-28b (Novagen) containing the histidine tag was replaced by the GST sequence from pET-14b GST-SF2/ASF (from Xiang-Dong Fu). An *Nde*I-*Bam*HI fragment containing the *prp2*<sup>+</sup> sequence in pPrp2/RK171a (from Judith Potashkin) was then inserted to generate pET-28b GST-*prp2*<sup>+</sup>.

**Production and Purification of Recombinant Proteins**—*Escherichia coli* strain BL21(DE3)pLysS (29) carrying the plasmid of interest was grown to an A<sub>600</sub> of 0.3–0.5 at 37 °C in LB containing 30 μg/ml chloramphenicol plus 30 μg/ml kanamycin for pET-28a *dsk1*<sup>+</sup> and pET-28b GST-*prp2*<sup>+</sup> or plus 50 μg/ml carbenicillin for pET-14b GST-SF2/ASF. The culture was induced in the presence of 0.4 mM isopropyl β-D-thiogalactopyranoside at 30 °C for 3 h (30). Cells were then centrifuged and washed with buffer A (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM MgCl<sub>2</sub>, 1 mM β-mercaptoethanol for histidine-tagged proteins or 1 mM dithiothreitol for GST fusion proteins). The cell pellet was resuspended at ~0.1 g of cells/ml or <10<sup>10</sup> cells/ml in buffer B (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5% glycerol, 5 mM EGTA for histidine-tagged proteins or 2 mM EDTA for GST fusion proteins, 1 mM β-mercaptoethanol for histidine-tagged proteins or dithiothreitol for GST fusion proteins) plus protease inhibitors (5 μg/ml of pepstatin, 5 μg/ml of chymostatin, 5 μg/ml of leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 25 μg/ml aprotinin). The cells were lysed by repetitive freezing and thawing followed by sonication using a sonicator (Polytron, Kinematica AG, Switzerland) for 30 s at setting 5. The lysate was centrifuged at 15,000 rpm for 15 min in an SA600 rotor (Sorvall) to separate the soluble fraction (supernatant) and inclusion bodies (pellet). The inclusion bodies were washed in buffer A, suspended in buffer B containing 1% Nonidet P-40 and the protease inhibitors (at 20–30 mg of the starting cell mass/ml of buffer), and sonicated.

His6-Dsk1 was purified from the soluble fraction by nickel-IDA agarose chromatography (31), whereas GST fusion proteins were purified from both soluble and inclusion body fractions by glutathione-agarose chromatography (32). IDA Sepharose Fast Flow (Pharmacia Biotech Inc.) was charged with 100 mM nickel chloride for 10 min at room temperature and washed extensively with water. The soluble fraction containing His6-Dsk1 was incubated with the nickel-IDA beads at 4 °C for 1 h with constant agitation in the presence of 5 mM EGTA and 10 mM imidazole. The protein-bound beads were packed in a column and washed with TBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 5 mM EGTA, 20 mM imidazole, and 0.1–0.5% Nonidet P-40. Nonidet P-40 was omitted during the final wash, and His6-Dsk1 was eluted from the column in TBS containing 150–200 mM imidazole. Similarly, GST fusion proteins were bound to glutathione-agarose beads, washed with TBS, and eluted in TBS containing 5 mM glutathione. Purified proteins were aliquotted, frozen in liquid nitrogen, and stored at –80 °C.

SRPK1 was a gift of Xiang-Dong Fu. SF2/ASF, SF2ΔRS, SRp30c, SRp40, SRp55, and Npl3 were gifts of Adrian Krainer.

**Antibodies**—A peptide corresponding to the COOH-terminal end of Dsk1, ATGEDVPGWATEIR, was conjugated to keyhole limpet hemocyanin, and the conjugate was used for immunization of rabbits (33). The anti-peptide antibodies were purified by affinity chromatography using the peptide coupled to Affi-Gel 15 resin (Bio-Rad) (34). mAb104 was isolated from hybridoma cells (American Type Culture Collection CRL 2067) (33). Anti-GST polyclonal antibodies were from Santa Cruz Biotechnology. Anti-SF2/ASF monoclonal antibody was from Adrian Krainer.

**Kinase Assay**—Purified kinase was incubated at 23 °C for 30 min with the substrate in a total volume of 20 μl containing a kinase buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol) in the presence of 50 μM ATP and 2 μCi of [γ-<sup>32</sup>P]ATP. The kinase reaction was terminated by boiling in SDS sample buffer, and the sample was resolved on a 10% or 12% SDS-polyacrylamide gel. Protein phosphorylation was detected by autoradiography. For Western blot analysis, the kinase reaction was performed by employing an ATP-regenerating system (10 mM creatine phosphate, 1 mM ATP, and 0.1 mg/ml creatine phosphokinase) without radioisotopes. Immunoblotting was performed as described (33, 35).

#### RESULTS

**The Dsk1 Kinase Has Distinct Substrate Specificity for SR Proteins**—Full-length Dsk1 protein was produced as a fusion construct with an NH<sub>2</sub>-terminal tag of 6 histidine residues (designated His6-Dsk1) in *E. coli*. The histidine-tagged Dsk1 protein in the soluble fraction of the bacterial lysate was bound to nickel-IDA agarose and eluted with buffers containing 150–200 mM imidazole. The His6-Dsk1 protein was recognized by polyclonal antibodies against its COOH-terminal peptide (ATGEDVPGWATEIR) as a 65-kDa protein (data not shown).

To assess whether the purified fusion His6-Dsk1 protein was catalytically active, we examined whether it was capable of phosphorylating MBP. Dsk1 was incubated with MBP in the presence of [γ-<sup>32</sup>P]ATP in a kinase buffer. As detected by gel electrophoresis and autoradiography, we observed that MBP was phosphorylated by the His6-Dsk1 (data not shown, but see Fig. 1, lane 10). Autophosphorylation of Dsk1 itself was detected after a longer exposure of the gel to x-ray film (data not shown; but see Fig. 2, lane 3), although the phosphorylation signal was much weaker than that of MBP. Both observations, which are consistent with our previous studies (7), indicate that the purified His6-Dsk1 is active as a kinase.

The protein sequence of Dsk1 suggests that it is homologous to human SRPK1, which is an SR protein-specific kinase (8). To test the functional similarity between the two proteins, we compared the substrate specificity between Dsk1 and SRPK1 in a cell-free kinase assay. As a reference, *Xenopus* Cdc2-human cyclin B complex (Cdc2-cyclin B) was used in parallel for comparison because all three kinases belong to a superfamily of serine/threonine-specific kinases (7, 8, 36). Cdc2-cyclin B complex is known as the maturation-promoting factor or the major M phase cyclin-dependent kinase for its function in inducing mitosis (37, 38). SR proteins are phosphoproteins *in vivo*

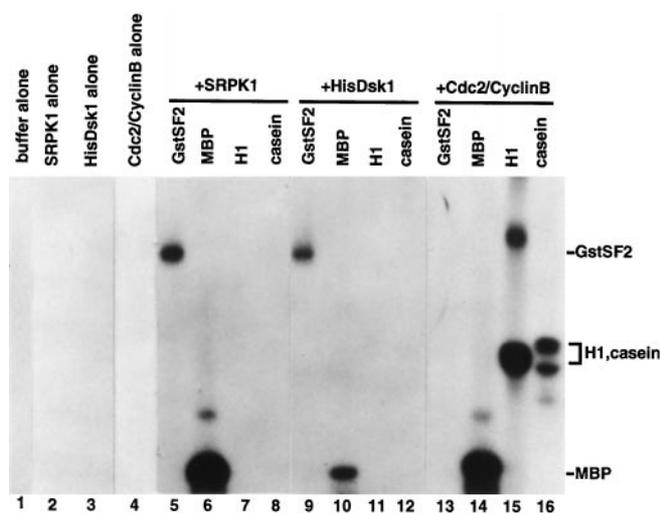


FIG. 1. Comparison of substrate specificity among SRPK1, Dsk1, and Cdc2 protein kinases. Purified SRPK1 (lanes 5–8), His6-Dsk1 (lanes 9–12), or Cdc2-cyclin B complex (lanes 13–16) was incubated with GST-SF2/ASF (lanes 5, 9, and 13), MBP (lanes 6, 10, and 14), histone H1 (lanes 7, 11, and 15), or casein (lanes 8, 12, and 16) in the presence of [ $\gamma$ - $^{32}$ P]ATP at 23 °C for 30 min. Samples were resolved on a 12% SDS-polyacrylamide gel and visualized with x-ray film. GST-SF2/ASF was added to a final concentration of 0.37  $\mu$ M, whereas MBP, histone H1, or casein was present at 10  $\mu$ M.

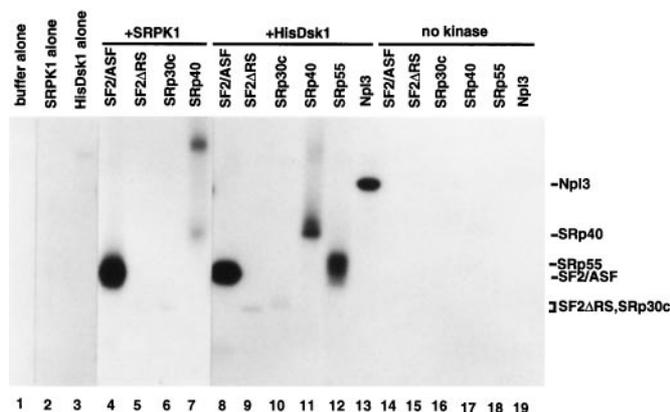


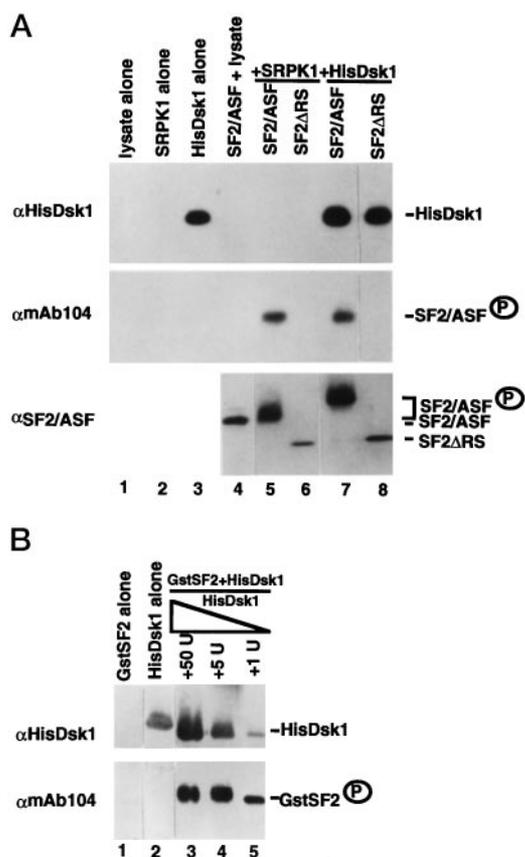
FIG. 2. Phosphorylation of various SR proteins by Dsk1 kinase. SRPK1 (lanes 4–7) or His6-Dsk1 (lanes 8–13) was incubated with various SR proteins, and samples were processed as described in Fig. 1. SF2/ASF protein was used as a positive control (lanes 4 and 8). SRp30c (lanes 6 and 10), SRp40 (lanes 7 and 11), SRp55 (lane 12), and SF2ΔRS (lanes 5 and 9) were tested at 0.5  $\mu$ M. Budding yeast Npl3 (lanes 13 and 19) was tested at 1  $\mu$ M. Each protein incubated without kinase served as a negative control (lanes 1–3 and 14–19).

and contain Cdc2 phosphorylation consensus sequences, (S/T)-P-X-(R/K); however, none of the SR proteins tested so far is an *in vitro* substrate for Cdc2 (8). Instead, SR proteins are phosphorylated by human SRPK1 and Clk/Sty in a highly specific and efficient manner (39, 40). Four proteins were tested as substrates in our assay: human SF2/ASF as an SR protein (39–41) in the form fused with GST (GST-SF2/ASF), bovine MBP, histone H1, and casein as serine/threonine-containing polypeptides. Histone H1 is typically used as a standard substrate for assaying the kinase activity of maturation-promoting factor (42). As expected, MBP, histone H1, and casein served as good substrates for the Cdc2-cyclin B complex (Fig. 1, lanes 14–16), whereas GST-SF2/ASF was not phosphorylated by Cdc2-cyclin B (Fig. 1, lane 13). In contrast, SRPK1 and His6-Dsk1 did not act on histone H1 or casein (Fig. 1, lanes 7, 8, 11, and 12). Dsk1 displayed a relatively moderate kinase activity toward MBP (Fig. 1, lane 10) compared with SRPK1 (lane 6) or

Cdc2-cyclin B (lane 14). Importantly, His6-Dsk1 phosphorylated GST-SF2/ASF protein very well (Fig. 1, lane 9), as did SRPK1 (lane 5). The specificity of SRPK1 and Dsk1 for GST-SF2/ASF is significant considering that GST-SF2/ASF (0.37  $\mu$ M) was present at a level that was at least 20-fold less than the other three polypeptides ( $\sim$ 10  $\mu$ M). Note that the concentrations of SF2/ASF and the other three substrates used in the assay are based on the standard assay conditions described previously (42, 43).

To examine further the specific activity of the Dsk1 kinase, we extended the list of substrates and included four additional human SR proteins in our assay (SF2/ASF, SRp30c, SRp40, and SRp55) (44). His6-Dsk1 phosphorylated SF2/ASF, SRp40, and SRp55 (Fig. 2, lanes 8, 11, and 12). In agreement, SRPK1 also phosphorylated SF2/ASF and SRp40 (Fig. 2, lanes 4 and 7). Interestingly, both kinases displayed very little activity toward SRp30c (Fig. 2, lanes 6 and 10). These results indicated the similarity between the two kinases in their substrate specificity. When SRp40 was used as substrate, an additional phosphorylated protein was detected which migrated in the gel considerably slower than SRp40 itself (Fig. 2, lanes 7 and 11). The nature of this high molecular weight protein is not clear. However, because SR proteins tend to aggregate without being phosphorylated (39), and the size of the slow migrating protein was about twice that of the recombinant SRp40, one likely explanation is that it might result from the association of two SRp40 proteins. The SRp55 used was a truncated version isolated from *E. coli*, which was missing a portion of its COOH-terminal domain (44). In addition to mammalian SR proteins, we also tested an SR protein from budding yeast *Saccharomyces cerevisiae*, Npl3 (39). As presented in Fig. 2, Npl3 is also a good substrate for His6-Dsk1 (lane 13). *In vivo* the majority of SR protein phosphorylation occurs on the serine residues in the RS domain (40). To test the importance of the RS domain for phosphorylation by Dsk1, a recombinant SF2/ASF with the RS region deleted (SF2ΔRS) was also used in the kinase assay (Fig. 2, lane 9). SF2ΔRS was not phosphorylated by SRPK1 (Fig. 2, lane 5; see Ref. 43). The level of phosphorylation by Dsk1 was reduced drastically to a barely detectable level when the RS region was deleted from SF2/ASF (Fig. 2, compare lanes 8 and 9). These results show that phosphorylation by Dsk1 requires an arginine/serine-rich region on the protein substrate.

**Phosphorylation by Dsk1 Generates a Phosphoepitope Shared by Native SR Proteins**—We showed above that Dsk1 phosphorylated various SR proteins having budding yeast to human origins with a specificity and efficiency similar to human SRPK1. SRPK1, as well as Clk/Sty, not only phosphorylates serine in the RS domain *in vitro*, but also the pattern of phosphorylation closely resembles that occurring *in vivo* (19). A unique feature of the phosphorylation by SRPK1 or Clk/Sty is the formation of a phosphoepitope within the RS domain, which can be recognized by mAb104 monoclonal antibody, which was found originally to recognize a specific phosphoepitope present in native SR proteins isolated from mammalian cells (45). To provide further direct biochemical evidence that Dsk1 is an SR protein-specific kinase, we asked whether Dsk1 produces the mAb104-reactive phosphoepitope upon phosphorylating SR proteins. In the experiment depicted in Fig. 3A, SF2/ASF or SF2ΔRS was incubated with Dsk1 or SRPK1 in the presence of an ATP-regenerating system. The samples were then analyzed by Western blotting with anti-Dsk1 antibody (Fig. 3A, top panel), mAb104 antibody (middle panel), or anti-SF2/ASF antibody (bottom panel) (lanes 5–8). Control samples were also analyzed in parallel (Fig. 3A, lanes 1–4). Note that probing the samples with anti-SF2/ASF antibody revealed that phosphorylation of SF2/ASF by both kinases caused a slight up-shift in gel mobil-



**FIG. 3. Dsk1-mediated phosphorylation of SF2/ASF protein generates the phosphoepitope specifically recognized by monoclonal antibody mAb104.** *Panel A*, SRPK1 (lanes 5 and 6) or His6-Dsk1 (lanes 7 and 8) was incubated with SF2/ASF (lanes 5 and 7) or SF2 $\Delta$ RS protein (lanes 6 and 8) in the presence of an ATP-regenerating system for 30 min at 23 °C. The samples were immunoblotted with anti-Dsk1 antibodies (top panel), mAb104 antibody (middle panel), and anti-SF2/ASF antibody (bottom panel).  $^{125}$ I-Protein A,  $^{125}$ I-labeled sheep anti-mouse antibodies, and alkaline phosphatase-conjugated goat anti-mouse IgG antibodies were used as the secondary antibodies in each panel, respectively. SF2/ASF was also incubated with a lysate from bacteria without plasmid (lane 4) to serve as a negative control. *Panel B*, different amounts of His6-Dsk1 protein (lanes 3–5) were added to the kinase reaction as in panel A to determine the linearity of the Dsk1 action. GST-SF2/ASF was incubated with 0.02  $\mu$ M (1 unit, lane 5), 0.1  $\mu$ M (5 units, lane 4), or 1  $\mu$ M (50 units, lane 3) His6-Dsk1 protein. The activity of Dsk1 was measured by immunoblotting with Anti-Dsk1 antibodies and alkaline phosphatase-conjugated goat anti-rabbit antibodies (top panel) and mAb104 antibody and alkaline phosphatase-conjugated goat anti-mouse IgG (bottom panel).

ity (Fig. 3A, bottom panel, lanes 5 and 7). Only two reactions in which full-length SF2/ASF was incubated with either SRPK1 or His6-Dsk1 resulted in the formation of an mAb104-reactive signal (Fig. 3A, lanes 5 and 7). Neither the unphosphorylated SF2/ASF (Fig. 3A, middle panel, lane 4) nor the SF2 $\Delta$ RS incubated with either kinase (lanes 6 and 8) was recognized by mAb104. Thus, the phosphoepitope generated by Dsk1 resided in the RS domain and was the same as that formed by SRPK1. These results allow us to argue strongly that Dsk1 is an SR protein-specific kinase in fission yeast.

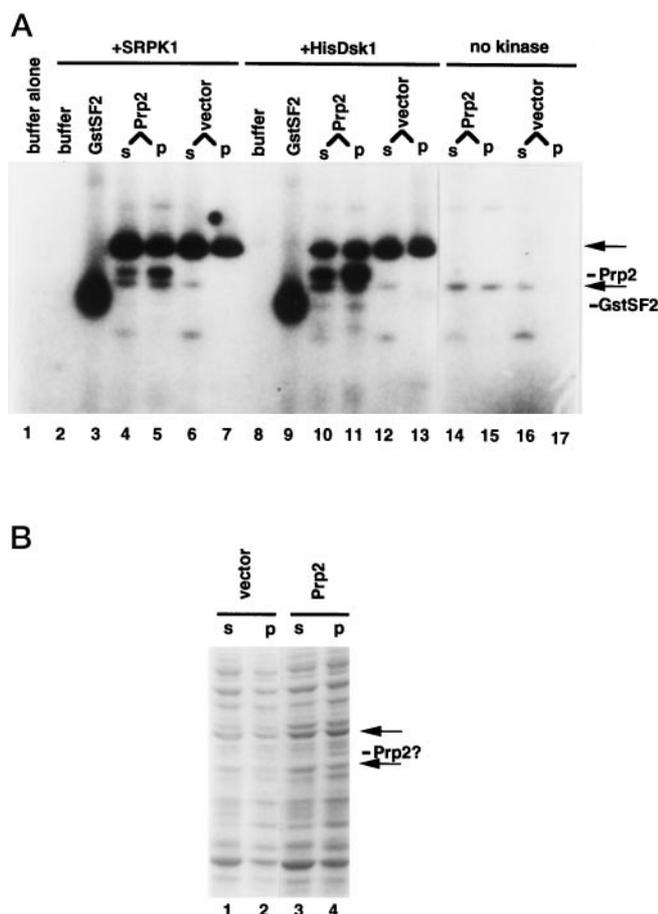
To show further that the recognition of SF2/ASF by mAb104 antibody is Dsk1-dependent, various amounts of Dsk1 protein (1, 5, or 50 units with 1 unit being 0.4 pmol) were incubated with SF2/ASF in a 20- $\mu$ l kinase reaction; the samples were then analyzed by Western immunoblotting (Fig. 3B, lanes 3–5). A concentration as low as 0.02  $\mu$ M His6-Dsk1 was sufficient to generate the mAb104-reactive phosphoepitope (Fig. 3B, lane 5, bottom panel), and the signal was increased substantially with

the concentration of Dsk1 at 0.1  $\mu$ M (lane 4). No further increase in intensity was observed when the concentration of His6-Dsk1 went up to 1  $\mu$ M (Fig. 3B, lane 3, bottom panel) probably because of the limited amount of GST-SF2/ASF protein used in the assay (0.74  $\mu$ M). Nevertheless, these results clearly show that the recognition of SF2/ASF protein by mAb104 antibody is Dsk1-dependent, and the extent of reaction is proportional to the amount of Dsk1 protein.

In summary, we have demonstrated using an *in vitro* system with purified components that the fission yeast Dsk1 has a kinase activity similar to that of human SRPK1. These data have provided the first direct biochemical evidence that Dsk1 specifically phosphorylates SR proteins in a fashion that closely resembles the phosphorylation of native SR proteins in mammalian cells. On the basis of these findings, we have established *in vitro* that Dsk1 is indeed a functional homolog of human SRPK1 in fission yeast.

*Dsk1 Phosphorylates the Fission Yeast Prp2 Protein in Vitro*—What is the *in vivo* target for Dsk1 in fission yeast? So far, Prp2 is the only protein identified in fission yeast which contains an RS domain and functions in pre-mRNA splicing (39). The protein sequence of Prp2 bears extensive similarity to mammalian splicing factor U2AF65 (25). The human U2AF65 has been shown to be a good substrate for both SRPK1 and Clk/Sty *in vitro* (8, 19). Therefore, we decided to test the phosphorylation of Prp2 protein by Dsk1 using the cell-free kinase assay system. Unlike the recombinant His6-Dsk1 and GST-SF2/ASF proteins, the expression level of recombinant Prp2 protein in *E. coli* was quite low (Fig. 4B, lanes 3 and 4). Thus, we examined whether the unpurified fission yeast Prp2 protein in the bacterial lysate could be phosphorylated by Dsk1. Lysates (soluble and inclusion body fractions) from bacteria transformed with a plasmid encoding the *prp2*<sup>+</sup> gene or the vector alone were incubated separately with SRPK1 (Fig. 4A, lanes 4–7) or His6-Dsk1 (lanes 10–13) in the presence of [ $\gamma$ - $^{32}$ P]ATP. A phosphorylated protein with the apparent molecular mass expected for Prp2 protein (~60 kDa) was observed specifically in the lysates from the bacteria containing the *prp2*<sup>+</sup> gene (Fig. 4A, lanes 4, 5, 10, and 11). Two bacterial proteins (one of which is indicated by the lower arrow in Fig. 4, A and B) were phosphorylated independent of SRPK1 or Dsk1. Interestingly, another bacterial protein (indicated by the top arrow in Fig. 4, A and B) was specifically labeled with  $^{32}$ P upon incubation with SRPK1 (Fig. 4A, lanes 4–7) or His6-Dsk1 (lanes 10–13). The identity of this bacterial protein was not investigated, but it provided an internal control for comparing the specific activity of the two kinases: the phosphorylation intensity of the bacterial protein was higher than that of Prp2 in the case of SRPK1 (Fig. 4A, lanes 4 and 5), but the intensity of the two was quite similar in the case of Dsk1 (lanes 11 and 12). These observations suggested that Dsk1 has a higher specific activity toward Prp2 than does SRPK1 and that Prp2 may be an *in vivo* substrate for Dsk1 in fission yeast.

To confirm that the phosphorylated 60-kDa protein was indeed the fission yeast Prp2 protein, we constructed and purified a recombinant GST-Prp2 fusion protein from *E. coli* (data not shown). Various amounts of purified GST-Prp2 protein (5–30 nM) were incubated with an excess amount of Dsk1 (100 nM) in the kinase assay (Fig. 5, lanes 6–10). Phosphorylation of GST-Prp2 by Dsk1 was quite efficient (phosphorylation was detected in the presence of 5 nM Prp2; Fig. 5, lane 10), and the level of phosphorylation increased with the increasing amount of GST-Prp2 protein used (lanes 6–10). In addition, the phosphorylation of Prp2 by Dsk1 was more efficient than that of SF2/ASF by Dsk1: the phosphorylation intensity of Prp2 at 25 nM concentration (Fig. 5, lane 7) was stronger than that of



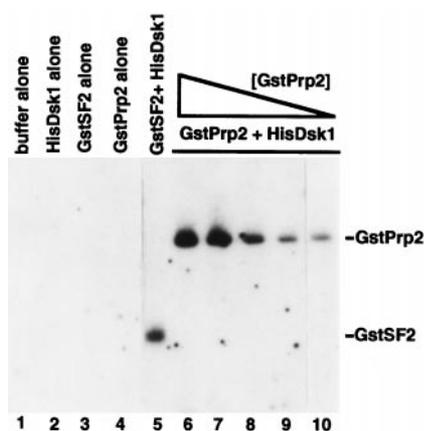
**FIG. 4. Dsk1 protein specifically phosphorylates fission yeast Prp2 protein.** Panel A, SRPK1 (lanes 2–7) or His6-Dsk1 (lanes 8–13) was incubated with lysates from bacteria carrying pRK171a (lanes 6, 7, 12, and 13) or pPRP2/RK171a encoding Prp2 (lanes 4, 5, 10, and 11) in the presence of [ $\gamma$ - $^{32}$ P]ATP at 23 °C for 30 min. Samples were separated on a 10% SDS-polyacrylamide gel and processed as in Fig. 1. Phosphorylation of purified GST-SF2/ASF by SRPK1 or His6-Dsk1 was used as a positive control (lanes 3 and 9). Both soluble (lanes 4, 6, 10, 12, 14, and 16) and inclusion body fractions (lanes 5, 7, 11, 13, 15, and 17) of the lysate were used. SRPK1 and His6-Dsk1 were omitted to monitor activities of bacterial endogenous kinases (lanes 14–17). The top arrow indicates the phosphorylation of one unidentified bacterial protein by SRPK1 or His6-Dsk1, and the lower arrow points to a bacterial protein that was phosphorylated by endogenous kinases. Panel B, expression of Prp2 protein in bacteria. Both soluble (lanes 1 and 3) and inclusion body fractions (lanes 2 and 4) of the lysate in panel A were subjected to electrophoresis on a 10% SDS-polyacrylamide gel and were stained with Coomassie Blue. Arrows indicate the putative bacterial proteins phosphorylated in panel A.

SF2/ASF at 60 nM.

Taken together, we showed that Dsk1 displayed higher kinase activity than human SRPK1 in phosphorylating Prp2, and Prp2 protein was phosphorylated by Dsk1 more effectively than SF2/ASF by Dsk1. In fact, Prp2 was the best substrate for Dsk1 among the variety of SR proteins tested in the *in vitro* assay system. These results are consistent with the notion that Dsk1 may directly act on Prp2 protein *in vivo*.

#### DISCUSSION

In this study, we have purified recombinant forms of both Dsk1 and Prp2/Mis11 proteins of fission yeast *S. pombe* and characterized the potential biochemical pathway linking these two proteins. Using a cell-free assay, we have demonstrated that Dsk1 phosphorylates SR proteins with high specificity, and Prp2 is the best substrate among various SR proteins tested. This study has provided the first molecular evidence



**FIG. 5. Kinase activity of Dsk1 on purified GST-Prp2 protein.** Dsk1 protein phosphorylates purified GST-Prp2 protein with high specificity and efficiency. His6-Dsk1 was incubated with GST-SF2/ASF protein (60 nM, lane 5) or purified GST-Prp2 protein at 30 nM (lane 6), 25 nM (lane 7), 15 nM (lane 8), 10 nM (lane 9), and 5 nM (lane 10). The experiment was performed as described in Fig. 1, and the phosphorylation was detected by autoradiography.

that Dsk1 is an SR protein kinase in fission yeast. These data also reveal the possible role of Dsk1 *in vivo* and suggest that Dsk1 may act directly on Prp2 protein by phosphorylation, thus affecting pre-mRNA splicing. In addition to its mitotic role in cell cycle regulation implicated by previous genetic studies, our results argue that Dsk1 may have dual functions in both pre-mRNA splicing and the cell division cycle.

Interestingly, many observations indicating the possible interplay between pre-mRNA splicing and the cell division cycle are obtained in fission yeast, more so than in budding yeast (for discussion, see Refs. 11 and 46). Out of 14 pre-mRNA processing (*prp*) mutants so far identified in fission yeast, 12 of them exhibit a cell division cycle (*cdc*) phenotype (11). One remarkable example is the two allelic genes, *cdc28* and *prp8*, which were independently discovered as a *cdc* and a *prp* mutant, respectively; yet each mutant displays both *cdc* and *prp* phenotypes (46). This may be attributed partly to the much higher intron content in the genome of *S. pombe* than that of *S. cerevisiae* (11). It is not surprising therefore that any abnormality in pre-mRNA splicing or in the regulation of pre-mRNA splicing may be manifested in fission yeast more readily than in budding yeast. Additionally, progression through the cell division cycle in fission yeast can be monitored easily by the size of the cell; therefore, perturbations in the cell cycle control points, particularly the G<sub>2</sub>/M phase transition, can be conveniently identified.

Although the present investigation has established that the fission yeast Dsk1 protein functions as an SR protein-specific kinase *in vitro*, several important questions remain to be addressed. What are the physiological consequences of the phosphorylation by Dsk1 in pre-mRNA splicing and cell cycle control? Is the role of Dsk1 protein in cell cycle regulation executed through the splicing pathway or by acting directly on the cell cycle control system at metaphase?

To answer these questions, it is necessary to determine *in vivo* targets of Dsk1 protein in fission yeast. Although *dis1* mutants are suppressed by increasing the expression level of *dsk1*<sup>+</sup> (7), the Dis1 protein is unlikely to be a substrate for the Dsk1 kinase because *dsk1*<sup>+</sup> also suppresses a null allele of *dis1*.<sup>1</sup> It is interesting to note that an intron is present in the *dis1* gene (13). On the other hand, the Prp2/Mis11 protein is a very attractive candidate as an *in vivo* substrate for Dsk1. Besides being phosphorylated *in vitro* by Dsk1 with high specificity based on our biochemical data, Prp2/Mis11 protein also

seems to possess a dual functional feature with respect to its role in both pre-mRNA splicing and the cell division cycle (see the Introduction). Although *prp2<sup>+</sup>* and *mis11<sup>+</sup>* are in the same gene, a gene homologous to the human splicing factor U2AF65, the effect of the *mis11*-453 mutation on pre-mRNA splicing has not been examined. To assess whether pre-mRNA splicing is defective, the *mis11* mutant was shifted to the restrictive temperature, and the total RNA was isolated for Northern blot analysis. A block of pre-mRNA splicing in the *mis11* mutant was detected<sup>3</sup> which is similar to that observed in *prp2* mutants (26). Thus, the *mis11*-453 mutation not only causes minichromosome loss at mitosis and cell growth block in interphase (27) but also abolishes pre-mRNA splicing at the restrictive temperature. The splicing defect observed in *mis11* mutant cells, together with previous genetic and cellular studies, again is consistent with our hypothesis that Dsk1 and Prp2/Mis11 protein may be interrelated in pathways connecting pre-mRNA splicing to the cell division cycle in fission yeast. It remains to be investigated whether there is a cause-consequence relationship between splicing and chromosome loss or whether Prp2/Mis11 uniquely has a dual role in these two cellular processes.

Could *dsk1* regulate pre-mRNA splicing and cell cycle progression through a pathway other than or in addition to affecting splicing activity *per se*? It is formally possible that an RS domain could also exist in proteins involved in other cellular processes in fission yeast, which are substrates for Dsk1. Interestingly, a large cyclophilin protein homologous to a natural killer tumor recognition protein has been found to contain an RS domain (47). Nonetheless, it is also conceivable that the splicing apparatus, as an indispensable part of proper nuclear architecture, may undergo stage-specific changes during the cell cycle; that is, the splicing machinery may be structurally and functionally distinct at mitosis and in the interphase. The Dsk1 protein may facilitate these changes in a cell cycle-dependent fashion. Specifically, at the metaphase/anaphase transition, Dsk1 may prepare the nuclear structure for the exit from mitosis by interacting with factors involved in chromosome segregation and components of the splicing machinery. This hypothesis would predict that if the concentration of Dsk1 protein in the cell is too high, the nuclear architecture may be retained in the interphase configuration and consequently prevent the cells from entering mitosis. Consistent with this theory, overexpression of the *dsk1<sup>+</sup>* gene leads to a delay at G<sub>2</sub>/M phase transition (7).

Cell viability is not affected by disruption of the *dsk1<sup>+</sup>* gene (7). It may simply reflect the fact that additional protein kinases exist in fission yeast with an overlapping function to Dsk1. Four kinases involved in SR protein phosphorylation have been identified, and two of them have been cloned (for review, see Refs. 39 and 40). In the mammalian system, it has been demonstrated that in addition to SRPK1, the Clk/Sty protein (Cdc28/Cdc2-like kinase) also phosphorylates SR proteins and controls the distribution of SR proteins within the nucleus (19, 43). The Clk/Sty family of protein kinases is conserved through evolution from yeast to human (19). The genome project of fission yeast reveals that the *ka23<sup>+</sup>* gene may be one of the members of this family based on the sequence similarity (48). Another candidate is the fission yeast Prp4 protein (49) which has been shown recently to phosphorylate human SF2/ASF protein *in vitro* (50). Because Dsk1 protein appears to be distributed in the cytoplasm during interphase (7), a Dsk1-like kinase may localize in the nucleus to act on SR proteins for efficient assembly of spliceosome and splicing of pre-mRNA in interphase. On the other hand, we cannot rule

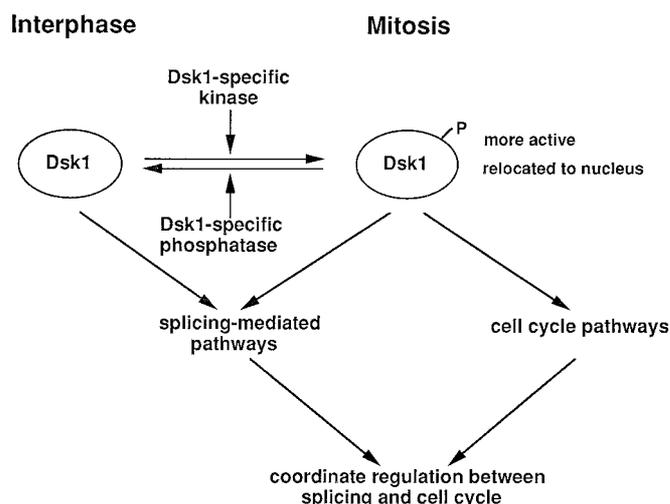


FIG. 6. A model for the fission yeast Dsk1 action. Dsk1 is hypophosphorylated in interphase and hyperphosphorylated at mitosis (7). The differential phosphorylation of Dsk1 may regulate the kinase activity, substrate specificity, or cellular localization of Dsk1 protein during the cell cycle. When relocated to the nucleus, Dsk1 protein may act on factors required for chromosome segregation at the metaphase/anaphase transition and components of the splicing machinery to facilitate the completion of mitosis. In addition, there may exist a Dsk1-stimulatory kinase and a Dsk1-inhibitory phosphatase to confer the changes of Dsk1 protein in cellular localization and activity during cell cycle. For details, see "Discussion."

out the possibility that in interphase a small amount of Dsk1 is present in the nucleus. In addition, Dsk1 protein is phosphorylated differentially during the cell cycle (7), which may exert another level of regulation.

To investigate further the function of Dsk1, we propose as a model that there may exist a regulatory network of protein kinases and phosphatases to couple pre-mRNA splicing to cell cycle control in fission yeast (Fig. 6). The network would consist of a family of Dsk1-like kinases (Dlks) specific for SR proteins, factors to regulate the kinases during the cell cycle, and their substrates in pre-mRNA splicing or in the cell cycle control pathway. As delineated briefly in the model (Fig. 6), the activity, substrate specificity, and intracellular distribution of Dsk1 may be altered by phosphorylation in a cell cycle-dependent manner. More specifically, upon phosphorylation, Dsk1 may favor one substrate over the other and thus, gain different or additional functions. Dsk1 may act by phosphorylating regulatory and structural proteins, such as splicing factors or cell cycle control elements, to closely coordinate pre-mRNA splicing with the cell division cycle. This hypothesis accommodates the possibilities that Dsk1 may have multiple substrates *in vivo*, and other Dsk1-like protein kinases may exist to overlap with Dsk1 function. New information revealing the mode of Dsk1 action will undoubtedly advance our understanding of the synchronous regulation between pre-mRNA splicing and the cell division cycle.

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