SRPK1 and Clk/Sty Protein Kinases Show Distinct Substrate Specificities for Serine/Arginine-rich Splicing Factors*

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Serine/arginine-rich (SR) proteins are essential for pre-mRNA splicing, and modify the choice of splice site during alternative splicing in a process apparently regulated by protein phosphorylation. Two protein kinases have been cloned that can phosphorylate SR proteins in vitro: SRPK1 and Clk/Sty. Here, we show that these two kinases phosphorylate the same SR proteins in vitro, but that SRPK1 has the higher specific activity toward ASF/SF2. SRPK1, like Clk/Sty, phosphorylates ASF/SF2 in vitro on sites that are also phosphorylated in vivo. Tryptic peptide mapping of ASF/SF2 revealed that three of the phosphopeptides from full-length ASF/SF2 phosphorylated in vitro contain consecutive phosphoserine-arginine residues or phosphoserine-proline residues. In vitro, the Clk/Sty kinase phosphorylated Ser-Arg, Ser-Lys, or Ser-Pro sites, whereas SRPK1 had a strong preference for Ser-Arg sites. These results suggest that SRPK1 and Clk/Sty may play different roles in regulating SR splicing factors, and suggest that Clk/Sty has a broader substrate specificity than SRPK1.

Serine/arginine-rich (SR)† proteins are essential splicing factors (1) that promote splice site recognition (2), and commit the spliceosome to a round of pre-mRNA splicing (3). cDNAs for nine human SR proteins have been cloned (1, 4–10); these proteins share the same overall structure, containing at least one RNA recognition motif (11) at their N terminus and an RS (arginine-serine-rich) domain at their C terminus (for review, see Ref. 12). Any individual SR protein is competent to rescue splicing of an SR protein-depleted extract, indicating a degree of redundancy in their function (1, 10). However, SR proteins have distinct specificities with regard to alternative splice site selection (3, 13). In each case, the splice site selected is dependent on the type and amount of SR protein present in the extract (3, 13). Changes in SR family expression levels have been seen at the transcript level during B cell development (14), insulin and mitogen induction (15), and T cell activation by phytohemagglutinin (16).

A cycle of phosphorylation-dephosphorylation is imperative for splicing to occur. Protein phosphatase 1 (PP1) either blocks spliceosome assembly at formation of the early complex (17) or switches splicing to the proximal 5′ splice site (18). Addition of phosphorylated SR proteins relieves this block in spliceosome assembly (17), implying that SR protein function is regulated by phosphorylation as well as by the level of expression. Whereas spliceosome assembly requires protein phosphorylation, catalysis of pre-mRNA splicing requires dephosphorylation steps to proceed. Inhibitors of protein phosphatase 2A (PP2A) block the first step of catalysis during which the 5′ splice site is cleaved, while inhibitors of PP1 and PP2A block both this step and the second step of 3′ splice site cleavage and exon ligation (19, 20).

Several SR protein kinases have been identified that phosphorylate ASF/SF2 within its RS domain. The first kinase activity identified, U1 70K kinase, is associated with the small nuclear ribonucleoproteins (21). The cDNA for U1 70K kinase has not been cloned. A second kinase, SRPK1, was cloned based on its ability to phosphorylate SC35 (22). A high level of SRPK1 inhibited splicing of 9-globin RNA in vitro (22). Clk/Sty (23, 24) was identified as an SR protein kinase after a two-hybrid screen using Clk/Sty as bait isolated several members of the SR family (25). Clk/Sty itself has an RS domain at its N terminus (25) placing it in the superfamily of serine/arginine-rich proteins (12).

Nuclear speckles are subnuclear structures where SR proteins are normally concentrated (26). Purified SRPK1 induced speckle disassembly in permeabilized cells (22). Similarly, overexpression of Clk/Sty in vivo resulted in disassembly of the nuclear speckles (25). SRPK1 and Clk/Sty are 32% identical across their kinase domain, including key amino acids that are likely to be involved in substrate specificity (25). Given that these related kinases phosphorylate ASF/SF2 in vitro and disassemble nuclear speckles in vivo, we sought to investigate the substrate specificities of these two protein kinases, to help elucidate their respective roles in vivo.

We show here that Clk/Sty and SRPK1 phosphorylate multiple SR proteins. Focusing on ASF/SF2, we demonstrate that both kinases phosphorylate ASF/SF2 in vitro on sites phosphorylated in vivo. SRPK1 displays greater specific activity toward ASF/SF2 than Clk/Sty, indicating that SRPK1 and Clk/Sty play different roles in vivo. In vitro, Clk/Sty phosphorylated more substrates, and more sites within ASF/SF2, than did SRPK1, indicating that Clk/Sty may play a broader regulatory role than SRPK1.
**Protein Purification and Antibody Production—** GST-ASF RS (25) and GST-Clk/Sty (27) were purified as described previously (25) except GST-Clk/Sty was eluted in 100 mM Tris, pH 7.5, 120 mM NaCl, 20 mM glutathione, 2 mM diethiothreitol, 2 mM benzamidine, and used directly in kinase assays. ASF/SF2, ASFARS, and the RS domain mutants of ASF/SF2 were purified from bacteria by nickel chromatography (4, 28, 29). SRPK1 was cloned into a baculovirus expression vector pAcSecG2T (Pharmingen) and expressed as a GST-fusion protein, which was purified the same way as GST-Clk/Sty. For substrate comparison between SRPK1 and Clk/Sty, ASF/SF2, SC55, Srp55, and U2AF65 were prepared from baculovirus as described previously (22). Histone H1 and myelin basic protein were purchased from Boehringer and Sigma, respectively. Anti-ASF/SF2 and anti-GST-ASF RS antibodies were prepared by immunizing New Zealand White rabbits individually with purified ASF/SF2 or GST-ASF RS.

**Peptide Synthesis and Purification—** Synthetic peptides (RSRS peptide, GRSRSHRSRS; SPRY peptide, RSPGPRSPRSRH) were prepared using conventional Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry with [2-(1H-benzoazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate activation on an Applied Biosystems 3H peptide synthesizer. Products were deprotected as outlined by the manufacturer, and the crude material was purified by reverse phase high pressure liquid chromatography. The authenticity of the peptides was confirmed by mass spectrometry and amino acid analysis.

**In Vitro Kinase Assays—** 100 nM GST-Clk/Sty (measured by Coomassie-stained gel using bovine serum albumin as a standard) and an equivalent kinase activity of GST-SRPK1 (determined by titration using ASF/SF2 as standard substrate) were incubated in kinase reaction buffer (40 mM Hepes, pH 7.5, 120 mM NaCl, 10 mM MgCl₂, 20 μM ATP, 2 mM diethiothreitol, 2 mM benzamidine, and 2 μM of [γ-⁴⁵P]ATP) for 30 min. The kinase reaction was terminated by boiling in equal volume of 2 × SDS sample buffer. Samples were separated on a 10% SDS-polyacrylamide gel, and the gel was exposed to autoradiography. For tryptic phosphopeptide maps, 600 nM ASF/SF2 or 500 μM peptide was added to the reaction. For comparison of substrates between GST-Clk/Sty and GST-SRPK1, 1 μM exogenous substrate was added. To test if ASF/SF2 is recognized by mAb104 after phosphorylation, 500 nM ASF/SF2, ASFARS, and GST-ASF RS were incubated plus minus 100 nM GST-Clk/Sty in kinase reaction buffer that contained 1 mM ATP and no [γ-⁴⁵P]ATP. These samples were resolved on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with 5 ml of mAb104 conditioned medium (30) or 1:250 dilution of either anti-ASF/SF2 or anti-GST-ASF RS antibodies. Immunoblots were developed by using enhanced chemiluminescence (Amersham).

**Determination of Relative Specific Activities of Clk/Sty and SRPK1—** The coding sequences of Clk/Sty and SRPK1 were fused with a FLAG tag sequence at their N termini, followed by cloning into the pSP73 vector from which the tagged kinases can be transcribed in vitro by T7 RNA polymerase (Promega). 0.5 μg of each plasmid was transcribed and translated in the TNT system (Promega) in a 25-μl reaction according to the manufacturer’s protocol. 2 μl of the reaction mixture was loaded on a 12.5% SDS-polyacrylamide gel followed by autoradiography for detecting in vitro translated product. The remaining reaction mixture was immunoprecipitated using 5 μg of an anti-FLAG monoclonal antibody (M2 from IBI) bound to protein G beads (Pharmacia Biotech Inc.) in 0.2 ml of Nonidet P-40 buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40). The beads were washed four times with the Nonidet P-40 buffer. The kinase reaction, on beads, was initiated by adding 20 μl of kinase reaction buffer containing 1 μM ASF/SF2. The reaction was incubated at 30 °C for 30 min. ³⁵S-Labeled in vitro translated kinases and ³²P-labeled ASF/SF2 bands were quantitated by phosphoimaging. Relative specific activity was calculated by dividing relative kinase activity with relative protein concentration.

**ASF/SF2 and Peptide Kinetics—** For kinetic comparison between Clk/Sty and SRPK1 in phosphorylating ASF/SF2, 100 nM GST-Clk/Sty and an equivalent kinase activity of GST-SRPK1 were used to phosphorylate five concentrations of ASF/SF2 (0.22 μM, 0.44 μM, 0.9 μM, 1.85 μM, and 3.7 μM). For peptide kinetics, sets concentrations of peptides (25 μM to 1 mM) were phosphorylated by GST-SRPK1 or GST-Clk/Sty in peptide reaction buffer (60 mM Hepes, pH 7.5, 10 mM NaF, 10 mM MgCl₂, 100 μM ATP, 2 mM diethiothreitol, 2 mM benzamidine, and 4 μl of [γ-³²P]ATP). Aliquots of the reaction were spotted on to P-81 paper, washed in 75 mM orthophosphoric acid, and counted in a liquid scintillation counter as described (31). The rate of phosphorylation was determined as picomoles of phosphate transferred/min. Kₘ and V_max were determined by linear regression using a Lineweaver-Burk plot.

**Comparison between SRPK1 and Clk/Sty in Phosphorylating RS Domain-containing Splicing Factors—** SRPK1 phosphorylates multiple members of the SR family (22). Only one member of the SR family, ASF/SF2, has been confirmed to be a substrate of Clk/Sty (25). To determine if Clk/Sty, like SRPK1, phosphorylates other SR proteins, several SR and SR-related proteins were tested as substrates for the two kinases. Using quantities of Clk/Sty and SRPK1 normalized to give equal level of ASF/SF2 phosphorylation, we found that both kinases phosphorylated the RS domain-containing splicing factors, SC55 (6), Srp55 (33), and U2AF65 (34), to similar levels (Fig. 1). Clk/Sty also phosphorylated histone H1 and myelin basic protein (Fig. 1 and Ref. 25). In contrast, SRPK1 had little activity on these substrates (Fig. 1 and Ref. 35). This result suggests that Clk/Sty has a broader substrate specificity than SRPK1.

Another difference between these two kinases was noted while normalizing their activities to give equal phosphorylation of ASF/SF2. SRPK1 appeared much more active for ASF/SF2 than Clk/Sty. Because SRPK1 and Clk/Sty were isolated from different sources, we sought a direct comparison between the two kinases. SRPK1 and Clk/Sty were in vitro translated as FLAG-tagged proteins (Fig. 2A). The translated proteins were immunoprecipitated using an anti-FLAG monoclonal antibody, and their kinase activities on ASF/SF2 were determined (Fig. 2B). Quantitative analysis showed that under these conditions, SRPK1 was 150-fold more active than Clk/Sty. Since SRPK1 and Clk/Sty had similar Kₘ values for ASF/SF2 (Table I), we postulate that SRPK1’s greater activity was due to an increase in K_cat. This result indicates that SRPK1 turns over ASF/SF2 faster than Clk/Sty. Perhaps the interaction of Clk/Sty’s RS domain with ASF/SF2 results in the slower release of ASF/SF2 (Fig. 2C).

**ASF/SF2 Phosphorylated by Clk/Sty Is Recognized by mAb104—** Although SRPK1 and Clk/Sty phosphorylate the same SR proteins, these polypeptides have many potential phosphorylation sites. The two kinases might, therefore, have identical, overlapping, or distinct substrate specificities. The monoclonal antibody mAb104 recognizes a phosphorylated epitope found on SR proteins (33). Bacterially expressed ASF/SF2 is recognized by mAb104 after phosphorylation in vitro by SRPK1 (35). To determine if bacterially expressed ASF/SF2 is reactive with mAb104 after phosphorylation by Clk/Sty, puri-
FIG. 2. Relative specific activities of Clk/Sty and SRPK1. A, wild type (Δt) and mutant (M) Clk/Sty and SRPK1 were in vitro translated as FLAG-tagged proteins. The mutant Clk/Sty contains a Lys → Arg change at position 190 (23, 27), and the mutant SRPK1 contains a Lys → Met change at position 109. Both mutations target the ATP binding site within each kinase. B, in vitro translated products were immunoprecipitated by an anti-FLAG antibody and subjected to a kinase assay using ASF/SF2 as substrate. The immunoprecipitated kinases (15S methionine-labeled) and in vitro phosphorylated ASF/SF2 (32P-labeled) were quantitated by phosphoimaging. Clk/Sty was immunoprecipitated 5 times more efficiently than SRPK1 in this experiment. Because the signal of wild type Clk/Sty is stronger than the mutant, possibly due to autophosphorylation, the relative protein level of immunoprecipitated Clk/Sty was based on the signal detected with the mutant kinase. The relative specific activities were determined by dividing the relative kinase activities by relative protein levels.

### Table I

**Kinetic parameters for ASF/SF2, RSRS, and SPRY after phosphorylation by Clk/Sty of SRPK1**

|        | $K_m$ (μm) | $V_{max}$ (pmol PO$_4$/min) | $V_{max}/K_m$ | RSRS:SPRY |
|--------|------------|-------------------------------|---------------|-----------|
| ASF/SF2-Clk/Sty | 0.40 | 0.20 | 0.50 |          |
| ASF/SF2-SRPK1 | 0.28 | 0.20 | 0.71 |          |
| RSRS-Clk/Sty 1 | 146.23 | 342.14 | 2.34 |          |
| RSRS-Clk/Sty 2 | 95.68 | 45.51 | 0.48 |          |
| RSRS-Clk/Sty 3 | 64.09 | 17.90 | 0.28 |          |
| RSRS-Clk/Sty final | 102.00 ± 41.43 |          |          |          |
| SPRY-Clk/Sty 1 | 124.87 | 62.80 | 0.51 | 4.60 |
| SPRY-Clk/Sty 2 | 236.90 | 22.46 | 0.01 | 5.02 |
| SPRY-Clk/Sty 3 | 61.83 | 3.35 | 0.54 | 5.15 |
| SPRY-Clk/Sty final | 141.20 ± 88.67 | 5.06 ± 0.48 |          |          |
| RSRS-SRPK1 1 | 25.00 | 45.40 | 1.82 |          |
| RSRS-SRPK1 2 | 61.03 | 4.10 | 0.07 |          |
| RSRS-SRPK1 3 | 78.30 | 3.86 | 0.05 |          |
| RSRS-SRPK1 final | 54.78 ± 27.19 |          |          |          |

Characterization of SRPK1 and Clk/Sty

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FIG. 3. Phosphorylation of ASF/SF2 by Clk/Sty and SRPK1. A, 500 nm ASF/SF2, ASF/SF2RS, or GST-ASF RS were incubated in an in vitro kinase assay plus or minus 100 nM Clk/Sty or SRPK1. The samples were separated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with mAb104. B, the same samples as in A were probed with anti-ASF/SF2 (leftmost four lanes) or anti-GST-ASF RS (rightmost two lanes).

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fied ASF/SF2 was subjected to an in vitro kinase assay in the presence or absence of Clk/Sty. Clk/Sty was able to generate the phosphopeptide recognized by mAb104 on full-length ASF/SF2 (Fig. 3A). Besides full-length ASF/SF2, we also tested two derivatives of ASF/SF2: ASFΔRS, which has the arginine/serine-rich (RS) domain deleted; and GST-ASF RS, which contains only the RS domain. Clk/Sty phosphorylates the RS domain of ASF/SF2 to similar levels as full-length ASF/SF2, but only weakly phosphorylates ASFΔRS (25). GST-ASF RS was recognized by mAb104 after phosphorylation by Clk/Sty indicating that the ASF/SF2 sites phosphorylated by Clk/Sty, and subsequently recognized by mAb104, lie within the C-terminal RS domains (Fig. 3A). ASFΔRS, on the other hand, was not recognized by mAb104 after incubation with Clk/Sty, indicating that this region does not contain the correct phosphopeptide or that Clk/Sty does not phosphorylate ASFΔRS to an extent where it is recognized by mAb104 (Fig. 3A). In vivo, SR proteins show altered migration on SDS gels due to multiple sites of phosphorylation (10). ASF/SF2 also displays reduced mobility after in vitro phosphorylation by SRPK1 (35). Purified bacterial ASF/SF2 and GST-ASF RS, but not ASFΔRS, exhibited a similar mobility shift after in vitro phosphorylation by Clk/Sty (Fig. 3B). Therefore, both kinases can phosphorylate ASF/SF2 on multiple sites, including the mAb104 phosphopeptide.

SRPK1 and Clk/Sty Phosphorylate Overlapping Sites on ASF/SF2—Tryptic phosphopeptide mapping has shown previously that sites on ASF/SF2 phosphorylated in vitro by Clk/Sty overlap sites phosphorylated on ASF/SF2 in vivo, indicating that Clk/Sty may regulate ASF/SF2 in vivo (25). To test whether SRPK1 phosphorylates ASF/SF2 at similar sites as Clk/Sty, we compared tryptic phosphopeptide maps of purified ASF/SF2 phosphorylated in vitro by SRPK1 or Clk/Sty. ASF/SF2 phosphorylated in vitro by either SRPK1 or Clk/Sty generated the phosphopeptides 1, 2, 3, 5, 6, 8, and F (Fig. 4, A, B, and D). The numbers refer to peptides present in ASF/SF2 phosphorylated in vitro in COS-1 cells, and the letters refer to peptides thus far seen only in ASF/SF2 phosphorylated in vitro by Clk/Sty (25) or SRPK1. The tryptic phosphopeptide maps of in vitro phosphorylated ASF/SF2 also revealed differences between SRPK1- and Clk/Sty-mediated phosphorylation. Peptides 7 and 9 were not seen following SRPK1 phosphorylation of ASF/SF2 and peptide G was not present in ASF/SF2 following phosphorylation by Clk/Sty (Fig. 4, A and B). Mixing of tryptic digests of ASF/SF2 phosphorylated in vitro by SRPK1 or Clk/Sty confirmed these results (Fig. 4C).

The extent of phosphorylation of two specific peptides in ASF/SF2 by SRPK1 or Clk/Sty differed. There was a decrease in peptide 5 phosphorylation and an increase in peptide 6 phosphorylation in ASF/SF2 phosphorylated by SRPK1 as compared to Clk/Sty (Fig. 4, A and B). It is possible that some spots (i.e., 6, E, and 7) may represent differentially phosphorylated states of the same peptide (36). If so, the lack of peptide 7 in ASF/SF2 phosphorylated by SRPK1 may represent a different stoichiometry of phosphorylation of a peptide rather than lack of that phosphopeptide. Neither kinase phosphorylated peptides 3, 4, and 10 that were present in ASF/SF2 phosphorylated in vivo, suggesting that other kinases may exist that also phosphorylate ASF/SF2 (25).

**SRPK1 and Clk/Sty Have Distinct Substrate Specificities for Peptides Derived from ASF/SF2 Sequence—Both SRPK1 and Clk/Sty phosphorylate ASF/SF2 within its RS domain (22, 25).**
argine at the P + 1 position although arginine was preferred. In contrast, SRPK1 was only able to phosphorylate serines followed by arginine. The $K_m$ for RSRS phosphorylated by SRPK1 (55 $\mu$M ± 27) was similar to that seen for Clk/Sty (Table I).

**Tryptic Mapping of SPRY and RSRS Phosphopeptides**—To determine if RSRS or SPRY sequences are phosphorylated in full-length ASF/SF2, the two peptides, after phosphorylation by Clk/Sty, were subjected to trypsin digestion and subsequent peptide mapping. The RSRS peptide produced two phosphopeptides (Fig. 5A) that appeared similar to peptides 8 and F of full-length ASF/SF2 phosphorylated by Clk/Sty (Fig. 5B). Further comparative analysis of RSRS and ASF/SF2 demonstrated that the phosphorylated RSRS tryptic fragments did co-migrate with peptides 8 and F from full-length ASF/SF2 (Fig. 5A) (5C and D). Both peptides were present in tryptic digests of ASF/SF2 phosphorylated by SRPK1 (Fig. 4A), consistent with the ability of SRPK1 to phosphorylate the RSRS peptide.

The SPRY peptide, phosphorylated by Clk/Sty, also produced two smaller phosphopeptides upon tryptic digestion (Fig. 5E), one of which co-migrated with peptide 5 of full-length ASF/SF2 (Fig. 5, D and F). This result suggests that phosphopeptide 5 of full-length ASF/SF2 is derived from the SPRY sequence. SRPK1, which could not phosphorylate the SPRY peptide, was able to phosphorylate peptide 5 in the context of full-length ASF/SF2, albeit less efficiently than Clk/Sty (Fig. 4A). Therefore, it appears that in full-length ASF/SF2, SRPK1 may phosphorylate serines followed by arginine (SP), although SP is not a good site in the context of a peptide. SRPK1 has higher affinity for full-length ASF/SF2 than for the RSRS peptide, which is consistent with SRPK1’s ability to recognize more sites within full-length ASF/SF2 (Table I).

Both SPRY and RSRS peptides produced tryptic fragments that overlapped peptide F of full-length ASF/SF2 (Fig. 5D). In a mix of RSRS and SPRY tryptic fragments, only three peptides were resolved, which is consistent with the SPRY and RSRS peptides overlapping at fragment F (Fig. 5D). Therefore, it is likely that peptide F is a mix of two different phosphopeptides that share similar electrophoretic properties.

**SRPK1 and Clk/Sty Differ in Their Ability to Phosphorylate Mutants of ASF/SF2**—To further investigate the catalytic properties of SRPK1 and Clk/Sty, we tested their ability to phosphorylate ASF/SF2 mutants that have substitutions in their RS repeats (29). SRPK1 phosphorylation of ASF/SF2 markedly decreased when all the serines in RS dipeptides were mutated to glycines (RG) or threonines (RT) consistent with the above observations that SRPK1 phosphorylated ASF/SF2 on serines in RS dipeptides (Fig. 6 and Ref. 35). The residual phosphorylation must have occurred on one or more of the four serines not mutated in the RG or RT variants, two of which are found in the SPRY peptide. Mutation of the arginines in RS dipeptides to lysines (KS) or glycines (GS), resulted in loss of phosphorylation of ASF/SF2 by SRPK1. This observation demonstrates that phosphorylation of any of the 20 serines within the RS domain of ASF/SF2 by SRPK1 requires the presence of arginine in the RS dipeptides. In the ASR mutant, the RS dipeptides were deleted but the other amino acids in the RS domain were retained. Similar to the KS and GS mutants, SRPK1 was unable to phosphorylate the ASR variant.

Phosphorylation of the various RS domain mutants by Clk/Sty revealed a different pattern than that observed for SRPK1. Phosphorylation by Clk/Sty also decreased when the RS dipeptides were mutated to RG, but not to the same extent as SRPK1, consistent with Clk/Sty phosphorylating the SPRY peptide. When the arginines in RS dipeptides were mutated to lysines in the KS mutant, ASF/SF2 phosphorylation by Clk/Sty showed only a slight decrease in level of phosphorylation. This
result indicates that Clk/Sty can accommodate lysine in place of arginine in its substrate binding site and is consistent with its broader substrate specificity. Unlike SRPK1, Clk/Sty phosphorylated both the GS and DS mutant proteins at a low level. In the DS construct, only the serines within the RNA binding domain and the four serines outside of the RS dipeptide repeats remain. From phosphopeptide mapping, the sites of phosphorylation of ASF/SF2 by Clk/Sty are limited to the RS domain (25). Of the four serines in the RS domain, two of the arginines surrounding serines 201, 227, and 234 were deleted. In contrast, the arginines surrounding serine 238 were retained. Therefore, it is possible that the minor level of phosphorylation of the GS and DS mutants by Clk/Sty occurs on serine 238 within the SPRY peptide sequence. Note that the RT mutant was not phosphorylated to the same level as wild type by either SRPK1 or Clk/Sty, indicating that threonine cannot substitute for serine as the phosphoacceptor.

**DISCUSSION**

**Phosphorylation of SR Proteins by SRPK1 and Clk/Sty—** Both SRPK1 and Clk/Sty phosphorylated multiple SR proteins in vitro, and neither kinase preferred one SR protein over another. SRPK1 weakly phosphorylated myelin basic protein (35) and was unable to phosphorylate ASFΔRS or histone H1 (Fig. 1 and Ref. 22). Although Clk/Sty preferred SR proteins as substrates, it also phosphorylated basic proteins such as histone H1 and myelin basic protein and weakly phosphorylated ASFΔRS (Ref. 25 and Fig. 1). Therefore, in vitro, Clk/Sty may phosphorylate proteins outside of the SR family and play a more general role in cellular regulation than SRPK1.

Consistent with SRPK1 being a more specific SR protein kinase, SRPK1 displayed greater specific activity toward ASF/SF2 as compared to Clk/Sty. This suggests that, in vivo, SRPK1 may be the more active SR protein kinase. However, Clk/Sty may be expressed at higher levels in cells or may require modification, such as phosphorylation, to become fully active.

SRPK1 and Clk/Sty Phosphorylate ASF/SF2 on Sites Phosphorylated in Vivo—Two methods were employed to determine if SRPK1 or Clk/Sty phosphorylate SR proteins on sites phosphorylated in vivo. One approach involved the use of mAb104 that recognizes a phosphoepitope found on SR proteins in vivo (33). Bacterially expressed ASF/SF2 was specifically recognized by mAb104 after phosphorylation by Clk/Sty, indicating that Clk/Sty phosphorylates this site(s) in vitro. Furthermore, mAb104 recognized the GST-ASF RS fusion protein following incubation with Clk/Sty, suggesting that the phosphorylated site recognized by mAb104 lies within the RS domain. Previous work has shown that SRPK1 phosphorylates ASF/SF2 in vitro at a site recognized by mAb104 (35). The finding that both SRPK1 and Clk/Sty generate a mAb104 phosphoepitope on ASF/SF2 suggests that they can phosphorylate similar sites. This observation was confirmed by phosphopeptide mapping. A tryptic map of ASF/SF2 phosphorylated in vitro by Clk/Sty generated 12 phosphopeptides, of which seven were also found in ASF/SF2 isolated from 32P-labeled cells (25). A phosphopeptide map of ASF/SF2 phosphorylated in vitro by SRPK1 was similar to the tryptic map of ASF/SF2 phosphorylated in vitro.
by Clk/Sty, except it lacked two peptides (seen in vivo) and contained an additional one (not seen in vivo). The position of peptide 7, which ASF/SF2 phosphorylated by SRPK1 lacks, is consistent with its being a lower phosphorylation state of peptide 6 that SRPK1 phosphorylates. Three important conclusions can be drawn from these data. First, SRPK1 phosphorylated ASF/SF2 on a set of peptides that overlapped with those phosphorylated in vivo, consistent with its being an SR protein kinase in vivo. Second, SRPK1 did not phosphorylate peptide 9 that was seen in vivo indicating that Clk/Sty or another kinase is likely responsible for phosphorylation of this site in vivo. Finally, peptides 3, 4, and 10 were present in ASF/SF2 phosphorylated in vivo, but not in ASF/SF2 phosphorylated in vitro by either kinase (25). Therefore, another SR protein kinase is probably responsible for these latter phosphorylation events. Possibilities for this kinase include the U1 70K kinase (21), cAMP-dependent protein kinase or protein kinase C (25), or another unidentified SR protein kinase.

**Clik/Sty and SRPK1 Display Distinct Substrate Specificities**—Two types of serines predominate in the RS domain of ASF/SF2: serines followed by arginine at the P+1 site or serines followed by proline. In the CDK2 and ERK2 crystal structures, an arginine forms the base of the P+1 binding pocket (37, 38). The bulkiness of the arginine side chain fills the P+1 binding pocket leaving room for only small amino acids, such as proline, at the P+1 site (37–39). This arginine is conserved in SRPK1 and Clik/Sty (25). To test if SRPK1 or Clik/Sty phosphorylate substrates with arginine or proline at the P+1 site, two peptides were synthesized: RSRS (GSRSRSRSRS) and SPRY (GRSPRYSPRHS). Both kinases preferred the RSRS peptide, suggesting that their P+1 binding pockets can accommodate arginine in contrast to CDK2 or ERK2. The preference for arginine at P+1 was more dramatic for SRPK1, as it was unable to phosphorylate the SPRY peptide. Although Clik/Sty preferred the RSRS peptide, it was competent in phosphorylation of the SPRY peptide, suggesting that Clik/Sty has fewer constraints than SRPK1 on the type of amino acid it can accommodate at the P+1 position.

To determine if residues contained within the RSRS and SPRY peptides are phosphorylated in full-length ASF/SF2, these peptides were subjected to trypsin digestion and peptide mapping. Both peptides generated two phosphorylated fragments that co-migrated with tryptic peptides contained within the full-length ASF/SF2. Based on these data, ASF/SF2 peptide 8 is apparently derived from an RSRS sequence, and peptide 5 from a SPRY sequence. Both phosphorylated peptides generated a second fragment that in each case migrated to a similar position as peptide F in full-length ASF/SF2. Since these two fragments must have distinct sequences, despite their similar migration properties, we cannot yet tell whether peptide F phosphorylated in vitro by SRPK1 or Clik/Sty contains one or both fragments.

The data discussed above indicate that both the RSRS and SPRY peptides contain sites phosphorylated in vivo, and in vitro by Clik/Sty. RSRS peptide 8 was present in ASF/SF2 phosphorylated by SRPK1 consistent with SRPK1 phosphorylating the RSRS peptide. Surprisingly, phosphopeptide 5 was present in ASF/SF2 phosphorylated by SRPK1 even though peptide 5 contained sequence from the SPRY peptide. However, this site was less prominent in full-length ASF/SF2 phosphorylated by SRPK1 than by Clik/Sty, indicating that it is a less preferred site for SRPK1. Since the SPRY peptide is not phosphorylated at all by SRPK1, we suggest that there are sites within full-length ASF/SF2, perhaps the RSRS stretch, that are required for SRPK1 to phosphorylate the SP motifs of peptide 5. An alternate explanation is that peptide 5 contains distinct co-migrating phosphopeptides that do not correspond to the SPRY peptide, and SRPK1 does not phosphorylate SPRY sequence at all.

**Clik/Sty Has a Broader Substrate Specificity than SRPK1**—When the RS dipeptides of ASF/SF2 were replaced by RG, phosphorylation of ASF/SF2 by SRPK1 and Clik/Sty was greatly reduced but not eliminated, indicating that either kinase can phosphorylate the four serines not found in an RS repeat. The higher level of phosphorylation of the RG ASF/SF2 mutant by Clik/Sty is consistent with Clk/Sty's ability to phosphorylate the SPRY peptide. Deletion of all the RS dipeptides or mutation of the arginine residues to glycines, resulted in loss of phosphorylation by SRPK1, and a decrease in phosphorylation by Clk/Sty, indicating that the arginines within these repeats are important for recognition of serines inside and outside of the repeats. The amino acids surrounding serine 238 were barely affected by the GS and ASR mutations, and the low level of phosphorylation of these mutants by Clk/Sty probably occurred on serine 238. Threonine cannot replace serine as a substrate for phosphorylation by either kinase. Of interest, Clk/Sty, but not SRPK1, phosphorylated the KS mutant to almost wild type levels. This result indicates that Clk/Sty only requires a basic charge for substrate recognition, whereas SRPK1 specifically requires arginine.

The function of SR proteins is thought to be controlled by phosphorylation. Indirect evidence suggests that the location of SR proteins and their function in spliceosome assembly is altered by phosphorylation (17, 22, 25, 40). In vitro, Clk/Sty phosphorylated more sites within ASF/SF2 than SRPK1. The last 24 amino acids that include all the SPRY sequence, but not the stretch of eight RS repeats, are dispensable for ASF/SF2 function in vitro (25). In contrast, deletion of the 16-amino acid RS stretch, or substitution of all the arginines or serines in this stretch to glycines, blocks the constitutive splicing function of ASF/SF2 (28, 29). Since the deletion of the SPRY sequence does not affect splicing in vitro, the significance of Clk/Sty phospho-
ylation of this sequence is unknown.

Another difference between the two kinases is the presence of an RS domain at the N terminus of Clk/Sty. This RS domain may provide Clk/Sty with a function in protein/protein interaction that SRPK1 lacks (2, 25, 41, 42). To this end, it should be noted that there is an isoform of Clk/Sty that contains only the RS domain (27).

In this study, we have compared the activities of these two protein kinases in vitro. In vivo, SRPK1 and Clk/Sty may be found in different cell types or in distinct locations within the cell. SRPK1 is most active during mitosis when splicing is shut down and SRPK1 inhibits splicing in vitro at high concentrations (22). It is not known whether Clk/Sty activity is regulated during the cell cycle. In vitro, Clk/Sty alters 5' splice site selection when overexpressed. Whether SRPK1's main role is to turn SR proteins off during mitosis and Clk/Sty's function is to control the activity of the SR family during alternative splice site selection remains to be determined. Further analysis is necessary to understand the functions of these kinases with respect to the regulatory role they play in pre-mRNA splicing and other processes.

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