Mass Spectrometric and Kinetic Analysis of ASF/SF2 Phosphorylation by SRPK1 and Clk/Sty*

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Assembly of the spliceosome requires the participation of SR proteins, a family of splicing factors rich in arginine-serine dipeptide repeats. The repeat regions (RS domains) are polyphosphorylated by the SRPK and Clk/Sty families of kinases. The two families of kinases have distinct enzymatic properties, raising the question of how they may work to regulate the function of SR proteins in RNA metabolism in mammalian cells. Here we report the first mass spectral analysis of the RS domain of ASF/SF2, a prototypical SR protein. We found that SRPK1 was responsible for efficient phosphorylation of a short stretch of amino acids in the N-terminal portion of the RS domain of ASF/SF2 while Clk/Sty was able to transfer phosphate to all available serine residues in the RS domain, indicating that SR proteins may be phosphorylated by different kinases in a stepwise manner. Both kinases bind with high affinity and use fully processive catalytic mechanisms to achieve either restrictive or complete RS domain phosphorylation. These findings have important implications on the regulation of SR proteins in vivo by the SRPK and Clk/Sty families of kinases.

It is estimated that more than one-half of human genes are alternatively spliced (1). Although many of these genes may contain only a few splice variants, a few have been shown to possess thousands (1–3). RNA splicing occurs in the nucleus at a macromolecular complex composed of many RNA and protein molecules known as the spliceosome (4, 5). In the last decade it has become apparent that both the assembly of the spliceosome and selection of splice sites depend on the proper phosphorylation of a family of splicing factors known as SR proteins (1). The SR proteins are so named because they have long stretches of arginine-serine dipeptide repeats in RS domains. Phosphorylation leads to translocation of the SR protein from the cytoplasm to the nucleus (6, 7) and recruitment of the SR proteins from sites of nuclear storage in speckles to nascent transcripts for splicing (8–10). Phosphorylated SR proteins are believed to facilitate both 5′ and 3′ splice site recognition through interaction with the RS domain in U1–70 K (a component of the U1 small nuclear ribonucleoprotein) and the U2AF heterodimer (11–13). More recently, RS domains were shown to interact directly with critical cis-acting elements in pre-mRNA (14, 15). In addition to their functions in the spliceosome, SR proteins are also shown to play a role in the export of processed mRNA from the nucleus to the cytoplasm for translation. Two shuttling SR proteins, ASF/SF2 and 9G8, have been shown to conduct the export of mRNA through interactions with a nuclear export factor, TAP (16–18). Hypophosphorylation of these SR proteins promotes interaction with TAP suggesting that transport may require splicing factor dephosphorylation. These shuttling SR proteins were also found to play a direct role in translation in the cytoplasm (19).

Although it is clear that SR proteins can be polyphosphorylated in the RS domain regions by the SRPK and Clk/Sty families of protein kinases (7, 20), the specificity and mechanism of SR protein phosphorylation by these kinases is still poorly understood. In previous investigations, we showed that SRPK and Clk/Sty have distinct substrate specificity and enzymatic kinetics in phosphorylating SR proteins (9). More recently, we demonstrated that SRPK1 uses a fully processive catalytic mechanism to phosphorylate the prototypical SR protein ASF/SF2 (21). This finding is interesting, because it indicates that SRPK1 makes a single encounter with ASF/SF2 and catalyzes the ATP-dependent phosphorylation of the RS domain without dissociating from the substrate. This unusual mechanism is prompted, in part, by a high affinity interaction between the enzyme and splicing factor (Kd ∼ 50 nM) (21). Once the SRPK1-ASF/SF2 complex is formed it dissociates very slowly in comparison to a fast forward rate for RS domain phosphorylation. Although a fully processive mechanism for serine phosphorylation is unique, it has been shown that the phosphorylation of tyrosine residues can occur in a similar manner. For instance, the tyrosine kinase Src phosphorylates Cas at numerous sites using a processive mechanism where no phosphotyrosine intermediates are released prior to complete phosphorylation of the substrate (22). In this case, it is thought that the SH2 domain of Src serves as a molecular tether for multiple rounds of tyrosine phosphorylation in the substrate, thereby, accounting for the absence of intermediate phosphoforms. In contrast, SRPK1 appears to use a unique docking site to anchor SR protein substrates near the catalytic site (23).

In this present study mass spectrometric and kinetic methods were used to determine how the SRPK and Clk/Sty families of protein kinases modify sequences in the RS domain of ASF/SF2. The results reveal that the RS domain in ASF/SF2 is not a random sequence with Arg-Ser repeats as the only recognizable feature. Instead, the RS domain can be divided into two subdomains, one of which was efficiently phosphorylated by SRPK1. In contrast, Clk/Sty was capable of transferring phosphate to all available serines in the RS domain. The phosphorylation of these subdomains appears to occur through similar catalytic mecha-

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6 The abbreviations used are: ASF/SF2, human alternative splicing factor; MOPS, 3-(N-morpholino)propanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; LysC, lysyl endopeptidase; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight mass spectroscopy; RS domain, domain rich in arginine-serine repeats; SR proteins, splicing factor containing arginine-serine repeats; SRPK, SR-specific protein kinase.
nisms. Both SRPK1 and Clk/Sty can phosphorylate the RS domain using fully processive mechanisms. Interestingly, SRPK1 phosphorylates only the N-terminal portion of the RS domain before dissociating, whereas Clk/Sty can stay attached and phosphorylate the entire RS domain.

**EXPERIMENTAL PROCEDURES**

**Materials**—ATP, MOPS, MES, MgCl₂, KCl, NaCl, acetic acid, sucrose, Kodak imaging film (Biomax MR), trifluoroacetic acid, bovine serum albumin, acetonitrile, and liquid scintillant were obtained from Fisher Scientific. Glutathione immobilized on 4% beaded agarose and free glutathione were purchased from Sigma. Precast 10% PAGE gels were obtained from Bio-Rad, and [γ-³²P]ATP was obtained from PerkinElmer Life Sciences. α-Cyano-4-hydroxycinnamic acid was obtained from Aldrich Chemicals and recrystallized once from ethanol.

**Expression and Purification of Recombinant Proteins**—Construction of pET19b-ASF/SF2 containing the ASF/SF2 coding sequence with a His₁₀ tag at the N terminus was constructed by inserting the PCR products of this gene in-frame into the NdeI and BamH1 sites of pET19 vector for expressing in *Escherichia coli*. The ASF/SF2 quadruple mutant (R210K/R218K/R229K/R240K) was generated by polymerase chain reaction using the QuickChange™ mutagenesis kit (Stratagene, La Jolla, CA) with pET19b-ASF wild-type construct as template. The plasmids for SRPK1 and Clk/Sty were constructed previously (24). A kinase-inactive form of SRPK1 (kdSRPK1) was generated by replacing Lys at position 109 with Met and was previously described (25). A kinase-inactive form of Clk/Sty (kdClk/Sty) was constructed by replacing Lys at position 190 with Met.

The plasmids for wild-type and mutant forms of His-tagged ASF/SF2 were transformed into the BL21(RIL) *E. coli* strain, and the cells were then grown at 37 °C in LB broth supplemented with 100 µg/ml ampicillin. Protein expression was induced with 0.1 mM isopropyl 1-thio-β-d-galactopyranoside at room temperature for 5 h. Cells were then pelleted and lysed by French Press using 10 ml of lysis buffer (0.1M MOPS, 10 mM Tris-HCl, pH 8.0, 0.3 M NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride). The insoluble fraction was collected by centrifugation at 14,000 rpm for 15 min, was applied to a 1.5-ml Ni²⁺ resin containing 20 mM imidazole. The protein was re-folded by passing through a column (8, 6, 4, 2, 1, and 0.5 M) and ASF/SF2 (5 and 10 µM) in a total reaction volume of 20 µl of SDS-PAGE loading buffer. A single peak was collected, dialyzed against 50 mM MOPS (pH 7.6), 500 mM NaCl, 1% Triton X-100, 1 mM EDTA, and 1 mM dithiothreitol, and stored at −80 °C. SRPK1 was purified using a previously published procedure (21).

**Phosphorylation Reactions**—The phosphorylation of ASF/SF2 by SRPK1 and Clk/Sty was carried out according to previously published procedures in the presence of 50 mM MOPS (pH 7.0), 10 mM free Mg²⁺, 5 mM bovine serum albumin, and [γ-³²P]ATP (600–1000 cpm pmol⁻¹) at 23 °C unless otherwise stated. Reactions were initiated with the addition of [³²P]ATP (250 µM) in a total reaction volume of 20 µl and then were quenched with 10 µl of SDS-PAGE loading buffer. A portion of each quenched reaction (20 µl) was loaded onto a 10% SDS-PAGE gel. Dried gels were then exposed with Kodak imaging film (Biomax MR), and protein bands corresponding to phosphorylated ASF/SF2 were excised and counted on the ³²P channel in liquid scintillant. Control experiments, specific activity determination, and time-dependent product concentrations were determined as previously described (21).

**Mass Spectrometric Analyses**—MALDI-TOF analyses were carried out using a PerSeptive Biosystems Voyager DE PRO spectrometer. Preparation of phosphorylated protein samples were typically carried out using SRPK1 (5 and 10 µM) and ASF/SF2 (5 and 10 µM) in the presence of 50 mM Tris-HCl (pH 7.4), 10 mM free Mg²⁺, 1 mM dithiothreitol at room temperature. Reactions were initiated with the addition of 3 mM ATP in a total volume of 40 µl. Reactions were then quenched with 3 volumes of 8 M urea, desalted with Zip-tip C₁₈, and eluted with 80% acetonitrile, 0.08% trifluoroacetic acid for MALDI-TOF analysis. Unphosphorylated sample controls were prepared in the same manner except ATP was omitted. The matrix solution consisted of 5 mg/ml α-cyano-4-hydroxycinnamic acid in 1:1 acetonitrile, ethanol, 0.52% trifluoroacetic acid. Final pH of the matrix solution was 2.0. LysC digestion of wild-type ASF/SF2 was carried out in a buffer containing 25 mM Tris-HCl (pH 8.5) and 1 mM EDTA. For the quadruple mutant of ASF/SF2, 0.01% SDS was added to the digestion buffer. All proteolysis reactions were allowed to progress for 18 h at 37 °C. In some cases, guanidine hydrochloride (3 M) was added to the digested samples prior to mass spectral analyses. The samples were then processed in the same manner as wild-type ASF/SF2.

**Data Analysis**—Progress curve data for ASF/SF2 phosphorylation were plotted as a ratio of incorporated phosphate and the total substrate concentration as a function of time and were fit to either a single- or double-exponential function unless otherwise stated. In the latter case, the amplitude of the first phase (α₁) represents the fraction of sites phosphorylated in the enzyme-substrate complex. The fraction of bound substrate was then calculated from the ratio of α₁ and the total amplitude (α₉₀₀) (Fraction bound = α₁/α₉₀₀). The dissociation constant for the enzyme-substrate complex (Kₐ) was determined by plotting the fraction bound as a function of [E]₀ and fitting the curve to Equation 1.

\[
BF = \frac{K_d + [S]_o + [E]_o - \sqrt{K_d^2 + [S]_o + [E]_o + 4[S]_o[E]_o}}{[S]_o}
\]

(Eq 1)

where BF is the fraction bound, and [S]₀ and [E]₀, are the total concentrations of ASF/SF2 and SRPK1. Experiments were typically performed using fixed [S]₀ and varying [E]₀.

**RESULTS**

**Phosphorylation Kinetics of ASF/SF2 by SRPK1 and Clk/Sty**—The phosphorylation of ASF/SF2 by multiple kinases was evaluated by excis-
Phosphorylation of ASF/SF2 by SRPK1 and Clk/Sty

**FIGURE 1.** Kinetics of ASF/SF2 phosphorylation by SRPK1 and Clk/Sty. A, progress curves for ASF/SF2 (20 nM) phosphorylation using 0.9 μM SRPK1 (●) and 0.5 μM Clk/Sty (○). The SRPK1 reaction is fit to a single exponential with an amplitude and a rate constant of nine sites and 1.5 min⁻¹, respectively. The Clk/Sty reaction is fit to a double exponential with rate constants of 1.4 and 0.29 min⁻¹ and amplitudes of 14 and 6 sites, respectively. B, MALDI-TOF spectra of ASF/SF2 with SRPK1. ASF/SF2 (10 μM) is mixed with SRPK1 (10 μM) for 1 h in the absence and presence of ATP (3 mM). The maximum for the M⁺1 peak increases from 30,351 in the absence of ATP (solid line) to 31,196 in the presence of ATP (dotted). C, MALDI-TOF spectra of ASF/SF2 with Clk/Sty. ASF/SF2 (10 μM) is mixed with Clk/Sty (10 μM) for 1 h in the absence and presence of ATP (3 mM). The maximum for the M⁺1 peak increases from 30,370 in the absence of ATP (solid line) to 32,087 in the presence of ATP (dotted line). D, progress curves for ASF/SF2 phosphorylation as a function of Clk/Sty. ASF/SF2 (20 nM) is phosphorylated using 60 (●), 90 (○), 180 (▲), and 900 (▼) Clk/Sty. The data at 60, 90, and 180 nM SRPK1 were fit to double exponential functions with amplitudes for the fast phase of 3.6, 5.1, and 6.3, respectively, and a common fast phase rate constant of 1.4 min⁻¹. The maximum amplitude change in all transients is 20. E affinity of ASF/SF2 for SRPK1 and Clk/Sty. Amplitudes of the initial, fast phase in the progress curves normalized to the total amplitude change are plotted as a function of total SRPK1 (●) and Clk/Sty (○) concentration. The data are fit using Equation 1 to obtain Kₐ values of 80 ± 20 nM for SRPK1 and 160 ± 33 nM for Clk/Sty.

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**Dissociation Constants of SRPK1 and Clk/Sty**—Because SRPK1 and Clk/Sty exhibit differential phosphorylation patterns, we sought to determine whether this unique specificity is linked to the stabilities of the enzyme-substrate complexes. To determine the affinities of both kinases for ASF/SF2, single turnover profiles were monitored as a function of total enzyme concentration. For both kinases, the phosphorylation kinetics of ASF/SF2 is biphasic at lower enzyme concentrations (Fig. 1, D and E), a phenomenon observed previously for SRPK1 (21). All progress curves approach a common end point defined by the total number of phosphorylation sites accessible by the kinase. In contrast, the kinetic profiles approximate single exponential transients that quickly reach an end point at high SRPK1 and Clk/Sty concentrations (>500 nM). The rate constants for the fast phases are similar in value and do not vary significantly over a wide range of enzyme concentrations (~1–2 min⁻¹). The amplitude of the first phase is used as an estimate of the initial concentration of the enzyme-substrate complex, and its enzyme dependence can then be used to determine the Kₐ values for both SRPK1 and Clk/Sty (Fig. 1F). Fitting the normalized amplitude data to Equation 1 provides Kₐ values of 80 and 160 nM for SRPK1 and Clk/Sty.

**Mapping the SRPK1 Phosphorylation Sites in ASF/SF2 by MALDI-TOF**—Because SRPK1, unlike Clk/Sty, phosphorylates only a subset of serine residues in the RS domain of ASF/SF2, we mapped the phosphorylation sites by MALDI-TOF using Lys-C-catalyzed proteolysis. This endoproteinase cuts specifically after lysine residues and, thus, is expected to produce ten fragments in ASF/SF2. Eight of these fragments are readily identified in the mass spectra, and four are displayed in Fig. 2 (A and B, -ATP). When ASF/SF2 is phosphorylated by SRPK1 (Fig. 2A, +ATP), a fragment (peptide 10) corresponding to the complete RS domain is absent, and no phosphorylated forms at higher molecular...
Phosphorylation of ASF/SF2 by SRPK1 and Clk/Sty

FIGURE 2. MALDI-TOF mass spectra of LysC-digested ASF/SF2. A, SRPK1 phosphorylation. MALDI-TOF spectra of a mixture of SRPK1 and ASF/SF2 in the absence (upper) and presence (lower) of ATP cleaved with LysC are shown with several peptide fragments identified. B, Clk/Sty phosphorylation. MALDI-TOF spectra of a mixture of SRPK1 and ASF/SF2 in the absence (upper) and presence (lower) of ATP cleaved with LysC are shown with several peptide fragments identified. Predicted LysC cleavage sites and large peptide fragments are shown in the top panel.

weight are detected. The disappearance of this fragment is likely due to poor flight of the phosphopeptide in the instrument (even after treatment with guanidine hydrochloride, see below), which is a widely known phenomenon in analyzing phosphopeptides by mass spectrometry (28). In contrast, the peaks corresponding to peptides outside the RS domain are unaffected by ATP treatment. A similar phenomenon is also observed when ASF/SF2 is phosphorylated by Clk/Sty and subjected to LysC proteolysis (Fig. 2B). Only the peak corresponding to the full RS domain disappears. These data indicate that both Clk/Sty and SRPK1 do not phosphorylate residues outside the RS domain.

Traditional protease mapping approaches to identify the subset of residues modified by kinases are not readily applicable here owing to the extensive arginine-serine dipeptide redundancy in the RS domain. To overcome this problem, four lysine mutations were introduced into the RS domain (R210K/R218K/R229K/R240K) to disrupt the arginine-serine repeat region and facilitate specific fragmentation of this domain by LysC, which was expected to generate five additional, unique peptide fragments in the MALDI-TOF spectrum (fragments 10a–c at the top of Fig. 3). In separate kinetic and mass spectrometric studies, we showed that SRPK1 phosphorylated the quadruple mutant to the same phosphorylation content and by the same mechanism as wild-type ASF/SF2 (data not shown). Upon phosphorylation of the quadruple mutant by SRPK1, two of the five fragments (10d and 10e) were unaffected while the remainder (10a–c) disappeared (Fig. 3A). To improve the flight of these fragments, guanidine hydrochloride (3 M) was added after LysC digestion to enhance detection (Fig. 3, B–D). For peptides 10a and 10c, new fragments were detected upon ATP treatment, which corresponded to the molecular weights of triple-phosphorylated peptides (Fig. 3, B and C). For peptide 10b, a peak corresponding to the quadruple-phosphorylated peptide was detected (Fig. 3D). Overall, 10 phosphates on these fragments were detected, a value consistent with the total phosphoryl content of the untreated quadruple mutant based on MALDI-TOF measurements (spectra not shown). These results, coupled with the absence of phosphorylation in peptides 10d and 10e, demonstrate that SRPK1 catalyzes site-specific phosphorylation in the N-terminal portion of the RS domain in ASF/SF2. Based on the position of serines within the peptide fragments, we conclude that SRPK1 modifies sites between residues 199 and 227.

Stepwise Phosphorylation of ASF/SF2 by SRPK1 and Clk/Sty—We showed previously that SRPK1 catalyzes ASF/SF2 phosphorylation in a processive manner in a pre-formed enzyme-substrate complex (21). To extend this analysis to a more natural kinase-substrate system, we determined whether processive phosphorylation is a result of the method of com-
plex preparation. To perform these studies, we combined native SRPK1 and ASF/SF2 without a re-folding step and determined whether this complex could be processively phosphorylated in start-trap experiments. In this experiment, the reaction is initiated with ATP (start) and simultaneously mixed with excess kdSRPK1 (trap). The experiment is performed under single turnover conditions where the SRPK1 concentration (1 μM) is ~12-fold above the $K_d$ for the complex ensuring that very little free ASF/SF2 is present at the time of start. If any phosphorylated forms of ASF/SF2 are generated and released from SRPK1 as expected in a distributive mechanism, kdSRPK1 will trap them and inhibit the reaction. However, if the reaction is processive, kdSRPK1 will not influence the progress curve. As shown in Fig. 5A, kdSRPK1 does not impact the phosphorylation of ASF/SF2 when added at the time of start in keeping with a processive reaction. In comparison, addition of kdSRPK1 to the complex prior to start with ATP leads to profound inhibition of the reaction progress curve indicating that kdSRPK1 is an effective trap for ASF/SF2. These data indicate that SRPK1 naturally catalyzes processive phosphorylation of ASF/SF2 in solution.

Clk/Sty Catalyzes Processive Phosphorylation of the Entire RS Domain—Because Clk/Sty phosphorylates ASF/SF2 to a greater extent than SRPK1, we wondered whether the complete modification of the RS domain could occur in a fully processive manner. To test for this possibility, we performed start-trap experiments using excess amounts of a kinase inactive form of Clk/Sty (kdClk/Sty) as a trapping agent for the detection of any free ASF/SF2. As shown in Fig. 5B, when added at reaction start, kdClk/Sty did not affect the reaction rate or end point for the Clk/Sty-catalyzed reaction. The efficiency of the trap was verified by demonstrating that kdClk/Sty could effectively inhibit the reaction when added to the complex prior to start. The initial velocity of the progress curve is lowered from 40 sites/min in the absence of kdClk/Sty, indicating that kdClk/Sty is an effective trap for ASF/SF2.

FIGURE 3. Mapping phosphorylation sites in ASF/SF2 using LysC digestion. MALDI-TOF spectra of a mixture of the quadruple ASF/SF2 mutant and SRPK1 before and after treatment with ATP are shown. Specific regions of the spectra are shown in the absence (A) and presence of 3 M guanidine hydrochloride treatment (B–D). Predicted peptide fragments resulting from LysC digestion in the RS domain (10a–10e) are shown in the top panel and appropriately labeled in the spectra. Phosphorylated forms of the peptides are designated in parentheses next to the fragment labels (1P–4P).
to 1.1 sites/min in the presence of pre-equilibrated kdClk/Sty, a rate change corresponding to a trapping efficiency of 97%. These findings indicate that the absence of inhibition in the start-trap experiment is not the result of poor binding of the inactive kinase trap. Overall, these findings suggest that Clk/Sty is capable of processively phosphorylating the entire RS domain.

Sequential Phosphorylation of RS Domain Segments By Clk/Sty—To determine whether Clk/Sty could sequentially phosphorylate portions of the RS domain, we analyzed the gel shift associated with ASF/SF2 phosphorylation. Unlike the SRPK1 reaction, the phosphorylation of ASF/SF2 by Clk/Sty occurs with a detectable gel shift in the phosphorylated species (inset to Fig. 6). A fast migrating species (lower) is populated early in the progress curve (−0.3 min) followed by the production of a slower migrating species (upper). The appearance and disappearance of the lower band were fitted to Equation 2,

\[
L = L_{\text{max}} \times \frac{k_1}{(k_2 - k_1)} \left[ \exp(-k_1 \times t) - \exp(-k_2 \times t) \right] \quad \text{(Eq. 2)}
\]

where \(L\) is the concentration of incorporated \(^{32}\)P normalized to the total ASF/SF2 concentration at any time \(t\), \(L_{\text{max}}\) is the maximum phosphoryl content of the lower band, and \(k_1\) and \(k_2\) are the rate constants controlling the production and disappearance of the lower band. The time dependence for the generation of the upper band was fitted to Equation 3,

\[
U = U_{\text{max}} \times \frac{1 - k_2 \exp(-k_1 \times t) - k_1 \exp(-k_2 \times t)}{(k_2 - k_1)} \quad \text{(Eq. 3)}
\]

where \(U\) is the concentration of incorporated \(^{32}\)P normalized to the total concentration of ASF/SF2 at any time \(t\), \(U_{\text{max}}\) is the phosphoryl content of the upper band, and \(k_1\) and \(k_2\) are defined as in Equation 2. Equations 2 and 3 are adapted from a previous study (29). As shown in Fig. 6, the appearance of the lower and upper bands follow a classic sequential reaction pattern where the lower (hypophosphorylated) species forms rapidly (3–6 min\(^{-1}\)) and then disappears at the same rate constant as the formation of the upper (hyperphosphorylated) species (~1 min\(^{-1}\)).
The total incorporation of $^{32}$P into both bands (dotted line in Fig. 6) approximates a single exponential relationship (1.5 min$^{-1}$) as observed previously under similar reaction conditions (Fig. 1E). The rapid production of the lower band and the lag in the generation of the upper band is consistent with the sequential phosphorylation of two regions in the RS domain of ASF/SF2. Finally, it is worth noting that this sequential determination implies an ordered succession of phosphorylation events but does not refer to whether this occurs in a processive or distributive manner. Rather, processivity is established in start-trap experiments and is independent of reaction sequence determination (Fig. 5).

**DISCUSSION**

In the present study, we characterized representatives of two major kinase families involved in post-translational modification of SR proteins. Enzyme-dependent single turnover data show that both kinases bind with similar affinities to ASF/SF2 (Fig. 1). By mass spectrometric mapping, we found, for the first time, that Clk/Sty is able to transfer phosphates to the majority of serine residues in the RS domain, whereas SRPK1 is highly restricted to a block of arginine/serine (RS) repeats in the N-terminal half of the RS domain, demonstrating clear regiospecificity between the two kinases (Figs. 2 and 3). The observation is consistent with previous phosphopeptide mapping, showing that the RS domain phosphorylated by Clk/Sty appears to give rise to a much more complicated phosphopeptide pattern than that by SRPK1 (9). These findings can now be used to explain some unexpected reactivities of SR proteins from other species. For example, the *Drosophila* ASF/SF2 ortholog dASF was shown to be a substrate for Clk/Sty, but not for SRPK1 (30). Sequence comparisons reveal that the first block of RS repeats in ASF/SF2 that are phosphorylated by SRPK1 is substituted with a glycine-tract in dASF, thereby, abolishing the SRPK1 sites but still preserving the Clk/Sty sites. The relaxed specificity of Clk/Sty-mediated phosphorylation indicates that this family of kinases may play a broad regulatory role in RNA metabolism in mammalian cells.

Partial Phosphorylation of the RS Domain by SRPK1—Although SRPK1 phosphorylates up to ten serines in the N-terminal portion of the RS domain, the kinetic transient associated with this modification is monophasic at high enzyme concentration. This raises the compelling question of how SRPK1 accomplishes multisite phosphorylation over a confined polypeptide stretch without introducing complex multiphasic kinetics. Two mechanisms can be invoked to account for this phenomenon. First, SRPK1 may not distinguish between these sites and consequently may phosphorylate them randomly in a tight complex with ASF/SF2. Such a mechanism would require considerable flexibility in the RS domain so that all sites are equally accessible to the kinase and, thus, kinetically indistinguishable. Second, SRPK1 may phosphorylate these residues in a sequential manner with the initial site representing the slow step in the overall reaction. In this mechanism, the RS domain may be more rigidly held in place so that SRPK1 starts at one locus and then phosphorylates in a single direction (either N- or C-terminal) until the entire block of residues is modified. The placement of a slow phosphorylation step early in the reaction cycle allows the overall reaction to appear monophasic, satisfying the observed single exponential kinetic traces at high enzyme concentrations (Figs. 1 and 5). At this time, it is difficult to ascertain which mechanism predominates, because it is unclear whether the SRPK1-catalyzed reaction has a defined directionality or is random. Nonetheless, given the electrostatic changes expected in the RS domain upon modification, it is more likely that phosphorylation would affect interaction of the RS domain with the active site of SRPK1 and, thus, give rise to more complex multiphasic kinetic behavior. Given this potential limitation, we suspect that SRPK1 is most likely to catalyze sequential, processive phosphorylation with an initial, rate-limiting priming step.

Modifying the Entire RS Domain without Enzyme Dissociation—Clk/Sty is not only capable of phosphorylating the entire RS domain of ASF/SF2, but it also accomplishes this feat using a fully processive pathway. Thus, Clk/Sty can remain attached to ASF/SF2 for a longer period of time, extending an otherwise regiospecific reaction and including all blocks of Arg-Ser repeats. Although it is not clear at this time whether either Clk/Sty or SRPK1 uses a preferred directionality for this modification, time-dependent gel shift assays suggest that regions of the RS domain are phosphorylated in sequential manners. For Clk/Sty an early phospho-intermediate is populated in the active site before conversion to the fully phosphorylated form. This early form is generated at a faster rate than the fully mature species, thus, establishing two identifiable blocks of Clk/Sty modification. Interestingly, Clk/Sty is capable of switching between these two phosphorylation blocks without dissociating from the SR protein.
Phosphorylation of ASF/SF2 by SRPK1 and Clk/Sty

Cellular Control of Phosphorylation Specificity—The current data indicate that Clk/Sty and SRPK1 can phosphorylate SR proteins sequentially but not simultaneously. Although Clk/Sty modifies additional residues in ASF/SF2 in the presence of equal amounts of SRPK1, an imbalance in the SRPK1 concentration can inhibit phosphorylation of the RS domain, because one kinase antagonizes the action of the other (Fig. 4). The cell avoids this potential conflict by anchoring the SRPK family of kinases in the cytoplasm (31) and restricting the Clk/Sty family of kinases to the nucleus (9). We have shown previously that the spatial control of SRPKs is achieved via a spacer within the conserved kinase core. Unlike SRPK1, Clk/Sty has much broader substrate specificity so the full potential of this enzyme family in splicing control is likely to be more complex. The methods outlined in this report to accurately assess phosphoryl content, regiospecificity of modification, and kinetic pathways are important for understanding the role of these SR proteins and their regulatory kinases in splicing regulation.

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