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Adaptable Molecular Interactions Guide Phosphorylation of the SR Protein ASF/SF2 By SRPK1

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Summary

The SR protein ASF/SF2, an essential splicing factor, contains two functional modules consisting of tandem RNA recognition motifs (RRM1-RRM2) and a C-terminal arginine-serine repeat region (RS domain). The serine protein kinase SRPK1 phosphorylates the RS domain at multiple serines using a directional (C-to-N-terminal) and processive mechanism, a process that directs the SR protein to the nucleus and influences protein-protein interactions associated with splicing function. To investigate how SRPK1 accomplishes this feat, the enzyme-substrate complex was analyzed using single and multi-turnover kinetic methods. Deletion studies revealed that while recognition of the RS domain by a docking groove on SRPK1 is sufficient to initiate the processive and directional mechanism, continued processive phosphorylation in the presence of building repulsive charge relies on the fine-tuning of contacts with the RRM1-RRM2 module. An electropositive pocket in SRPK1 that stabilizes newly phosphorylated serines enhanced processive phosphorylation of later serines. These data indicate that SRPK1 uses stable, yet highly flexible, protein-protein interactions to facilitate both early and late phases of processive phosphorylation of SR proteins.

Keywords

kinase; kinetics; phosphorylation; splicing; SR protein

The splicing of precursor (pre-mRNA) is essential for proteome diversity and many cellular regulatory processes but is also associated with numerous diseases when mistakes are propagated into the mature, spliced mRNA.^{1; 2; 3; 4} Splicing occurs in a macromolecular complex known as the spliceosome, a dynamic assembly of five small nuclear ribonucleoproteins (snRNPs) and many protein cofactors.⁵ An important family of splicing

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cofactors is the SR proteins so named because they contain a C-terminal domain composed largely of arginine-serine dipeptide repeats (RS domain). In addition to these repetitive sequences, SR proteins contain one or two N-terminal RNA recognition motifs (RRMs) that are essential for recognition of exonic enhancer sequences in pre-mRNA.^{6; 7; 8¶} The SR proteins play roles in both constitutive and alternative splicing.^{9; 10} While SR proteins are critical for the early stages of spliceosome assembly during the establishment of the proper 5' and 3' splice sites in the pre-mRNA,^{11; 12} they are also thought to connect spliced mRNA to RNA export machinery and modulate protein translation in the cytoplasm.^{13; 14; 15} SR proteins are now becoming recognized as potentially important targets for cancer therapy as the prototypical SR protein, ASF/SF2, was recently identified as a proto-oncogene with the observation that it is overexpressed in many tumors and transforms immortalized rodent fibroblasts by altering the splicing pattern of critical tumor suppressor and cell-cycle regulatory genes.⁴

Phosphorylation of the RS domains of SR proteins is important for the biological function of these splicing factors at several levels. The serine-specific protein kinase SRPK1 phosphorylates multiple sites in the RS domain of the SR protein, ASF/SF2, a modification that promotes binding to an import receptor, Transportin-SR, and delivers ASF/SF2 to the nucleus.^{16; 17; 18; 19} SRPK1 & its homolog SRPK2 are unique among the serine kinases in that they will not phosphorylate threonine and their kinase cores are bisected by a large insert domain (approx 250 aa).²⁰ While its function is not fully understood, the insert domain localizes the kinase to the cytoplasm where it can modify and support regulated nuclear entry of SR proteins.²¹ Once in the nucleus the phosphorylated RS domain of ASF/SF2 assists in the interaction of the SR proteins with U1-70K (a component of the U1 snRNP) and the U2AF heterodimer at the 5' and 3' splice sites, respectively.^{11; 12} A second family of protein kinases typified by Clk/Sty further alters the phosphoryl content of ASF/SF2 and impacts spliceosomal function as both hypo- and hyperphosphorylation of ASF/SF2 by this kinase can inhibit in vitro splicing reactions.²² While some SR proteins are exclusively localized to the nucleus others such as ASF/SF2 and 9G8 are shuttling SR proteins since they can move from the nucleus to the cytoplasm in a phosphorylation-dependent manner. It has been demonstrated that dephosphorylation of the shuttling SR proteins ASF/SF2 and 9G8 facilitates binding to the nuclear export protein TAP.¹⁵

In previous studies we showed that SRPK1 modifies the N-terminal portion of the RS domain (RS1) of ASF/SF2 using a processive mechanism in which the enzyme stays attached for about

¶Abbreviations:

ASF/SF2	human alternative splicing factor
LysC	lysyl endoproteinase
MALDI-TOF	Matrix assisted laser desorption ionization-time of flight mass spectroscopy
RS domain	domain rich in arginine-serine repeats
RRM	RNA recognition motif
SR protein	splicing factor containing arginine-serine repeats
SRPK	SR-specific protein kinase

8 cycles of rapid serine phosphorylation before releasing the splicing factor.^{23; 24} SRPK1 performs this feat using an ordered mechanism in which the kinase initiates phosphorylation in the center of the RS domain near the RS1/RS2 boundary (Fig. 1A) and attaches phosphates onto serines moving in a general C-to-N-terminal direction.²⁵ We recently solved an X-ray structure for SRPK1 with a monophosphorylated form of ASF/SF2 lacking RRM1 and RS2.²⁶ Although portions of the RS1 segment are not visible in this model, several important features that help define the enzyme-substrate interface are now apparent. First, the N-terminal portion of RS1 (N'-RS1) resides in a docking groove made up of a long electronegative channel in the large lobe of SRPK1 (Fig. 1B). This interaction would place the C-terminal portion of RS1 in the active site for initial phosphorylation near the RS1/RS2 boundary. Second, although RRM1 is not present in the structure, RRM2 makes three well-defined interactions with SRPK1 including one contact with two residues from the glycine-rich loop and several additional contacts in helices α D and α F (Fig. 1B). Third, an electropositive pocket is positioned to interact with a phosphoserine from the substrate. The presence of this phospho-serine supports the experimental finding that the kinase initiates phosphorylation near the C-terminus of RS1²⁵ and suggests that nearby phospho-serines in the RS domain may be stabilized by this electropositive pocket as the reaction progresses.

In cases where X-ray structures are now available, the addition of a single phosphate does not induce large changes in the conformation of the enzyme-substrate complex. For example, no significant changes in the kinase domain of protein kinase A or its bound substrate are observed upon phosphorylation of a single serine in the active site.²⁷ Such observations raise the question of how protein kinases might accommodate a sequential phosphorylation reaction where the substrate must 'slide' through the active site. Glycogen synthase kinase-3 (GSK3) catalyzes polyphosphorylation of its protein substrates using an electropositive pocket that recognizes P +4 phosphoserines and imposes a C-to-N-terminal phosphorylation direction.²⁸ Although the X-ray structure of the SRPK1-ASF/SF2 complex reveals potentially important contacts for the initiation of phosphorylation near the RS1/RS2 border, how sequential phosphorylation events are facilitated in the RS1 segment of the RS domain while the substrate remains bound is not known. In this present study we address the role of three critical groups of contacts made between the enzyme and substrate that could be important for controlling reaction dynamics. We found that SRPK1 uses its electronegative docking groove to grip the N-terminal end of RS1, thereby, initiating processive and directional phosphorylation that begins near the RS1/RS2 border. Contacts made between the kinase and the RRM2s are not essential for initial recognition and early stages of the processive reaction but instead play a vital role in conjunction with the electropositive pocket to facilitate the rate and processive modification of later serines in the reaction. Overall, the data indicate that SRPK1 uses multiple, choreographed contacts to provide both flexibility and stability for the RS domain as it traverses the active site.

Results

RS Domain Is Sufficient for High Affinity Binding of ASF/SF2

To define core structural elements involved in high affinity ASF/SF2 binding, we made a series of N-terminal deletions in the substrate (Fig. 2A). These deletion constructs were made with a common C-terminal His tag. To measure binding affinities of these constructs we used a competition assay in which the phosphorylation rate of a fixed amount of ASF/SF2-His (50 nM) is monitored as a function of varying concentrations of a competing substrate. Since the assay is performed under low phosphorylation levels (<10%), the inhibitory parameters of the competitor substrate (K_i) reflect the binding affinity of its unphosphorylated form. The inhibition of ASF/SF2-His phosphorylation is shown in Fig. 2B as a function of increasing competitors ASF(Δ RRM1)-His and ASF(Δ RRM12)-His. The time-dependent

phosphorylation of ASF/SF2-His is presented as relative velocity plotted against the total competitor concentration. The apparent K_I values are then converted to true K_I values using equation (1) and the K_m for the fixed substrate. The K_I for ASF/SF2-His was measured using a fixed amount of ASF(Δ RRM1)-His and varying wild-type protein ($K_I = 90$ nM). In general, removal of the RRM s including the linker segment between RRM2 and RS1 did not reduce binding affinity by more than 2-fold (legend to Fig. 2B).

To ensure that removal of the RRM s does not impact binding mode, we performed steady-state kinetic analyses using varying ASF/SF2-His and varying, fixed amounts of an RRM-deleted substrate. In double reciprocal plots, we showed that ASF(RS12)-His, the most dramatic deletion, functioned as a competitive inhibitor of ASF/SF2-His phosphorylation (Fig. 2C), suggesting that the wild-type and RRM-deleted substrate bind in a similar manner. To demonstrate that domain removal did not impact RS domain positioning in the active site, the phosphoryl contents of the substrates were measured using MALDI-TOF analyses. As shown in Fig 2D, removal of both RRM s did not impair phosphorylation compared to wild-type (14 sites). Finally, to control for any potential effects of the affinity label, we studied the binding of N-terminally tagged forms of ASF(Δ RRM1), ASF(Δ RRM12), and wild-type substrate and found that they display similar K_I values as the C-terminally tagged substrates (data not shown). Overall, the combined data indicate that the RS domain is sufficient for high affinity binding to SRPK1.

RS Domain Can Initiate But Not Sustain Processive Phosphorylation

The above deletion analyses indicate that the RRM s may not participate in the phosphorylation mechanism. To determine whether the RRM s could modulate SR protein phosphorylation, we performed kinetic analyses of several mutants lacking either RRM1 [ASF(Δ RRM1)], RRM2 [ASF(Δ RRM2)] or both RRM s [ASF(Δ RRM12)]. In single turnover experiments, SRPK1 rapidly attaches about 12 phosphates onto ASF/SF2 (4 min^{-1}) and then adds two phosphates in a much slower phase (0.13 min^{-1}) (Fig. 3A). Removal of either RRM1 or both RRM s lowers the amplitude and increases the rate of this initial phase. In comparison, removal of RRM2 does not affect the amplitude but does increase the rate constant for the initial phase. Taken together, the kinetic data indicate that the RRM s are not necessary for efficient phosphorylation of the RS domain.

To determine whether the RRM s influence processivity, we performed start-trap experiments (Fig. 3B). In this assay, SRPK1 ($1 \mu\text{M}$) is preequilibrated with substrate (250 nM) prior to the addition of ^{32}P -ATP ($100 \mu\text{M}$) in the absence and presence of excess kinase-inactive SRPK1 (kdSRPK1) which contains a single Lys-to-Met substitution in the ATP binding site.²⁴ If phospho-intermediates are released from the active site, as expected in a non-processive (i.e.-distributive) reaction, kdSRPK1 will bind them and inhibit reaction progress. Thus, the number of serines modified prior to reaction inhibition reflects the extent of processive phosphorylation. For ASF/SF2, ASF(Δ RRM1), ASF(Δ RRM2) and ASF(Δ RRM12), SRPK1 attaches about 8, 6, 7, and 3 phosphates, respectively, prior to dissociation and reaction inhibition by kdSRPK1 (Fig. 3B-F). To ensure that kdSRPK1 can trap free phospho-intermediates, a trap-start control experiment is employed in which kdSRPK1 is added prior to the addition of ^{32}P -ATP. Since kdSRPK1 substantially inhibits the initial reaction velocity in these experiments, the inability to trap early phospho-intermediates in start-trap experiments is not due to poor binding of the trapping agent. The low linear rates in the trap-start experiments reflect a small level of substrate not trapped by kdSRPK1 and is used as a background in fitting the amplitude of the start-trap experiments. Overall, the data imply that the RRM s, while not necessary for binding and initial processivity, are important for maintaining processive phosphorylation of ASF/SF2 in the later phase of the reaction (i.e.- after the first three serines).

High Affinity ASF/SF2 Binding Is Resistant to RS Domain Charge Alterations

Since the RS domain is sufficient for high affinity binding of unphosphorylated ASF/SF2 (Fig. 2), we next addressed which regions in the domain constitute this binding. We made charge-to-Ala substitutions in 7 individual blocks encompassing the entire RS domain and linker segment of ASF/SF2 (Fig. 4A). Based on MALDI-TOF analyses, the mutant proteins were substrates for SRPK1 with Blocks 1 through 4 and Blocks 2/3 and 3/4 being phosphorylated to a similar level as ASF/SF2 (~14 sites). Block 1/2 was phosphorylated at a lower extent (8 sites) suggesting that large removal of positive charge in RS1 has some impact on phosphorylation content (data not shown). Blocks 1 through 4 bind with high affinity to SRPK1 in competition assays, suggesting that ASF/SF2 can tolerate 5-6 consecutive charge replacements anywhere in the RS domain without impacting binding affinity (Fig. 4B). However, larger charge mutations had a significant impact on binding mode. Unlike the smaller block mutants, the larger block mutations were not capable of completely inhibiting this reaction (Fig. 4C). For example, although Block 1/2, Block 2/3, and Block 3/4 initially lowered the phosphorylation rate of ASF(Δ RRM1) in a concentration-dependent manner similar to other mutants, 76, 57 and 20 % of the activity remained at high competitor concentrations, respectively. These results are consistent with partial competitive inhibition²⁹ and suggest that SRPK1 can bind well to the larger block mutants and still support the phosphorylation of ASF(Δ RRM1), the control substrate. This mode of inhibition is shown in Scheme 1 where S is ASF(Δ RRM1) and I is one of the large block mutants. The data are fit to equation (2) for partial competitive inhibition to obtain K_I and αK_I values of 40 and 60 nM for Block 1/2, 45 and 100 nM for Block 2/3 and 120 and 800 nM for Block 3/4, respectively. Overall, the results indicate that the block mutants, although lacking many positively charged residues (5-11 Arg/Lys), can still bind with high affinity to SRPK1 as evident in low K_I values.

Since the RS domain can accommodate numerous charge changes with minimal impact on affinity, we investigated the phosphorylation of simple Arg-Ser peptides [(RS)₈ & (RS)₁₆] to determine a minimum number of arginines necessary for high affinity binding. In competition studies, we found that while (RS)₈ binds with greatly reduced affinity (K_I =1.6 μ M), (RS)₁₆ binds with a K_I close to that for ASF/SF2 (Fig. 4D). For (RS)₁₆, the ability to lower the velocity of ASF(Δ RRM1) phosphorylation to near zero indicates that this peptide does not use a partial competitive mechanism (Fig. 4). In contrast, we found no plateau with (RS)₈ in the velocity vs. competitor plot up to 4 μ M (data not shown in plot) with no evidence for partial competitive inhibition. The data indicate that while SRPK1 can recognize an Arg-Ser stretch of 8 repeats, high affinity binding requires more positively charged residues. Overall, the combined data indicate that while the RS domain is sufficient for high affinity binding, regions outside this domain are likely to contribute to binding when charge in the RS domain is removed.

Redundant Binding Determinants Revealed Through C-Terminal Deletions

Although the N-terminal deletions suggest that the RS domain is the principal component for high affinity binding of ASF/SF2 (Fig. 2), its affinity is highly resilient to large charge changes (Fig. 4). To investigate whether regions in the RRM also contribute to binding therefore explaining this resiliency, we studied RS domain mutants (Fig. 5A). If the RS domain contains all necessary residues for initial ASF/SF2 binding, then ASF(Δ RS) should bind very poorly, if at all. Interestingly, we found that ASF(Δ RS2) and ASF(Δ RS) bind with K_I values that are only 2-fold higher than that for wild-type ASF/SF2 (Fig. 5B). Unlike the large charge block mutants, these deletions lower the velocity to near zero. Although ASF(Δ RS) is not a substrate for SRPK1 as expected, ASF(Δ RS2) is phosphorylated at 12 sites based on MALDI-TOF analyses (data not shown). Since the RRM1-RRM2 module is not necessary for binding, we investigated the binding mode of ASF(Δ RS). In steady-state kinetic analyses, ASF(Δ RS) behaves as a competitive inhibitor against wild-type ASF/SF2 (Fig. 5C), suggesting that the two molecules occupy common binding sites on SRPK1. These findings indicate that the

RRM1-RRM2 domain pair in wild-type ASF/SF2 is bound to SRPK1 and that ASF(Δ RS) can compete for regions in the enzyme that bind these domains, indirectly displacing the RS domain. Finally, although ASF(Δ RS2) is phosphorylated to a slightly lower extent than wild-type ASF/SF2, SRPK1 processively phosphorylates about 8 serines, in line with the wild-type substrate (Fig. 5C). These findings suggest that while the RS domain is sufficient for high affinity interactions, other surfaces on SRPK1 have the capacity to bind the RRM2 to compensate for partial loss of high affinity binding mediated by the RS domain.

Since a construct lacking the RS domain, ASF(Δ RS), could readily inhibit the ASF/SF2 phosphorylation we next investigated whether the individual RRM1 and RRM2 are capable of binding to SRPK1. We designed two deletion constructs that express only RRM1 [ASF(RRM1)] or RRM2 with the linker segment [ASF(RRM2)] (Fig. 5A) and measured their abilities to inhibit ASF/SF2 phosphorylation in the competition assay. Unlike the high affinity RRM1-RRM2 construct, ASF(RRM1) and ASF(RRM2) bind poorly to SRPK1 displaying elevated K_d 's of 1.6 and 6.3 μ M, values that are between 18- and 73-fold higher than that for the full-length substrate. These data indicate that the high affinity of the RRM1-RRM2 construct relies not on the presence of one but on both RRM1 and RRM2. To investigate whether RRM1 could interact directly with SRPK1 or simply stabilize the conformation of RRM2 and its interactions with the enzyme, we made 4 alanine substitutions in RRM2 (W134, Q135, E184, R154) that constitute the SRPK1-RRM2 interface based on the X-ray structure.²⁶ The high affinity of this mutant construct, ASF(4M- Δ RS), suggests the contacts in RRM2 are flexible and/or the RRM1 directly interfaces with SRPK1 (Fig. 5C). Overall, the C-terminal deletion studies reveal that ASF/SF2 presents two separable modules, RRM1-RRM2 and the RS domain, that can independently bind SRPK1 with high affinity yet the separate domains within the RRM1-RRM2 module can function cooperatively in enhancing substrate binding.

Docking Groove in SRPK1 Controls Initiation of Processive Phosphorylation

Since the RS domain confers high affinity binding and commitment to about 3 processive phosphorylation steps (Figs. 2 & 3), we wished to address whether specific charged residues in the docking groove that contact the RS1 portion serve to initiate the processive reaction. We made six charge-to-alanine mutations in the docking groove of SRPK1 (Fig. 6A). While four of these residues directly interact with N'-RS1 (D564, E571, D548, & K615), two are nearby and are anticipated to interact with RS1 (E558, 557). These mutations result in a modified kinase [SRPK1(6M)] with impaired catalytic power. In single turnover kinetics, the initial fast phase rate and amplitude for SRPK1(6M) are about 4-fold (4 vs 1 min⁻¹) and 2-fold (12 vs. 6 sites) lower than that for wild-type (Fig. 6B). Despite these kinetic changes, SRPK1(6M) incorporated the same amount of ³²P in ASF/SF2 after 25 minutes as wt-SRPK1 in single turnover progress curves (data not shown) suggesting that mutation does not impact phosphoryl content. In competition assays we found that docking site mutation reduced binding affinity by only 2-fold (Fig. 6C). To determine whether the docking groove affects the phosphorylation mechanism, start-trap experiments were performed. In these assays, we found that SRPK1(6M) phosphorylates ASF/SF2 using a strictly distributive mechanism (Fig. 6D). The phosphorylation of only one site in the presence of kdSRPK1 relative to the trap-start control is likely the result of slow exchange of the unphosphorylated substrate relative to forward catalysis. However, after the first site is phosphorylated, the SR protein dissociates and is trapped by kdSRPK1. These findings suggest that the docking groove, while not important for overall phosphoryl content or binding affinity, plays a vital role in controlling the initiation of efficient processive phosphorylation.

Electropositive Pocket in SRPK1 Assists Late Phosphorylation Steps

While processive phosphorylation is guided at different stages by the RS domain and RRM1-RRM2 module contacts, other factors may contribute to this reaction. To determine whether

the electropositive pocket in SRPK1 stabilizes phosphorylated forms of ASF/SF2, a series of mutations in the pocket that fix the phospho-serine in SRPK1 were made (Fig. 7A). We made a single alanine mutation at Arg-561 in helix α G [SRPK1(RA)], a double alanine mutation at Arg-515 & -518 in the P+1 loop [SRPK1(2RA)], and a triple alanine mutation encompassing all three residues in the P+1 loop and helix α G [SRPK1(3RA)]. None of these mutations had a significant impact on overall binding affinity or phosphoryl content of ASF/SF2 (Fig. 7B & C). Instead, the mutations had a significant effect on the kinetics of ASF/SF2 phosphorylation. In single turnover experiments, the mutations did not affect the initial phosphorylation rate but significantly lowered the amplitude of the fast phase (Fig. 7C). Whereas wt-SRPK1 rapidly phosphorylates the first 12 sites, all three mutants lowered this initial phase amplitude to about 5-7 sites. Start-trap experiments were also performed to determine the mechanism of phosphate addition. As shown for the triple mutant, SRPK1(3RA), the reaction is largely processive within the fast, initial phase (5 sites) of the reaction (Fig. 7D). Owing to the slower rate of the secondary phase and the intrinsic background rate of the trap-start control, we cannot determine whether the slower phase of the reaction is also processive. Nonetheless, the data show that the electropositive residues play no role in controlling reaction processivity in the early phase of the reaction where the first 5 serines are phosphorylated. However, the pocket is very important for facilitating the rate of later phosphorylation steps.

Docking Groove Regulates Initiation & Directional Phosphorylation

Since the docking groove in SRPK1 is essential for initiating processive phosphorylation of the RS domain, we next investigated whether this region could also regulate the phosphorylation order. We showed previously that SRPK1 prefers to initiate phosphorylation toward the C-terminal end of RS1.²⁵ To investigate whether the docking groove or the electropositive pocket of SRPK1 could impact directionality, we determined whether SRPK1 (6M) and SRPK1(3RA) catalyze ordered phosphorylation. For this, we employed an ASF/SF2 construct [ASF(5R1K)] with a single Arg-to-Lys mutation near the center of RS1 (Arg-214) and several Lys-to-Arg mutations in RRM2 that can be separated into two fragments using the endoprotease LysC (Fig. 8A). We showed previously that phosphorylation of ASF(5R1K) with wt-SRPK1 followed by LysC treatment generates N- (19 kDa) and C-terminal (5 kDa) fragments whose ratio is close to unity based on the phosphorylation regiospecificity in RS1. In ATP limitation experiments, ASF(5R1K) (0.25 μ M) is added to wt-SRPK1 (1 μ M) and varying, limiting amounts of ³²P-ATP (0.1-50 μ M) and allowed to react to completion (20 min) before the sample is cleaved by LysC (Fig. 8B). In control experiments, we showed that increasing the incubation time does not alter overall phosphoryl content indicating that the reactions reach a true endpoint. The data indicate that the phosphorylation of the C-terminal fragment precedes the N-terminal fragment, consistent with a directional, C-to-N terminal mechanism. While SRPK1(3RA) show a similar trend as that for wt-SRPK1, SRPK1(6M) shows more extensive phosphorylation in the N-terminal fragment at low ATP concentrations, suggesting that C-terminal modification is not strongly favored for this mutant. At each ³²P-ATP concentration, the total phosphoryl content of ASF(5R1K) is calculated in control experiments lacking LysC and the ratio of N- and C-terminal fragments is plotted as a function of phosphoryl content (Fig. 8C). For both wt-SRPK1 and SRPK1(3RA), phosphorylation in the C-terminal fragment is favored strongly (~100-fold) at the reaction start while SRPK1(6M) displays a significantly reduced preference (~5-fold).

Since the above ATP limitation experiments suggest that SRPK1 docking residues are important for ASF/SF2 phosphorylation order, we wished to establish whether this phenomenon measured under equilibrium conditions was also observed under kinetic conditions. To address this, we measured the order of substrate phosphorylation using pulse-chase experiments in which the enzyme-substrate complex is reacted with ³²P-ATP (1 or 100 μ M) in an 8 second pulse phase before addition of excess cold ATP (10 mM) in the chase

phase. A low ^{32}P -ATP level of $1\ \mu\text{M}$ was used to ensure that the chase phase would capture the initial phase of the reaction ($\leq$ phosphorylation site). For wt-SRPK1, very little N-terminal phosphorylation is observed after the pulse phase using $1\ \mu\text{M}$ ^{32}P -ATP compared to $100\ \mu\text{M}$ ATP consistent with ordered, C-to-N-terminal phosphorylation of RS1 (Fig. 8D). Although similar results are observed for SRPK1(3RA), extensive phosphorylation of the N-terminal fragment is observed at $1\ \mu\text{M}$ ^{32}P -ATP for SRPK1(6M) compared to $100\ \mu\text{M}$. Whereas a low N/C ratio of 0.06-0.07 was attained after the first phosphorylation site (at $1\ \mu\text{M}$ ^{32}P -ATP) for wt-SRPK1 and SRPK1(3RA), an N/C ratio of 0.40 for SRPK1(6M) was achieved when less than one site (0.2 site) is modified at $1\ \mu\text{M}$ ^{32}P -ATP. These findings are consistent with the ATP limitation experiments and indicate that the docking groove plays a vital role in regulating phosphorylation initiation at the RS1/RS2 border.

RRM2 Contacts Affect Processive But Not Directional Phosphorylation

The deletion analyses suggest that ASF/SF2 requires an RRM directly N-terminal to the RS domain for processive phosphorylation of later serines (Fig. 3). To address whether the position of this RRM and its contacts with SRPK1 serve a unique kinetic role, we studied how SRPK1 phosphorylates an ASF/SF2 construct with a docking-defective RRM2. We made four alanine substitutions in RRM2 that disrupt contacts with SRPK1 [the same as in ASF(4M- Δ RS)] and studied this quadruple mutant, ASF(4M), using single turnover analyses. ASF(4M) is phosphorylated to the same extent as wild-type ASF/SF2 and displays similar kinetic parameters as the RRM-deleted substrates, namely the initial phase is faster than wild-type substrates ($4\ \text{vs.} >10\ \text{min}^{-1}$) (Fig. 9A). Removal of the residues leads to a reduction in processive phosphorylation relative to wild-type ASF/SF2 (8 vs. 5 sites) (Fig. 9B), by an amount larger than the single RRM1 and RRM2 deletions but smaller than removal of both RRMs (Fig. 3F). Consistent with the deletion results, unphosphorylated ASF(4M) binds with similar affinity as wild-type ASF/SF2 (data not shown). The combined data indicate that disruption of the RRM2 contacts impacts the phosphorylation mode of later serines in a manner intermediate between single RRM1 or RRM2 removal and complete deletion of both RRMs. These findings suggest either that there may be some flexibility in the way RRM2 interacts with SRPK1 or that RRM1 and its N-terminal position serves some role in processive phosphorylation. The latter is supported by the observation that removal of RRM1 in a deletion construct lowers processivity from 8 to 6 sites (Fig. 3F).

To address whether RRM2 contacts serve a role in directional phosphorylation, we introduced the four alanine mutants from ASF(4M) into the cleavage vector ASF(5R1K). This new mutant protein, ASF(4M-5R1K), binds with similar affinity to SRPK1 and is phosphorylated to the same extent as wild-type ASF/SF2 (data not shown). Disruption of the RRM2 contacts does not appear to alter the normal C-to-N-terminal preferred reaction (Fig. 9C). At low phosphoryl content ($0.2\ \mu\text{M}$ ATP), SRPK1 prefers to phosphorylate the C-terminal fragment by a factor of about 100, similar to that for wild-type ASF/SF2 (Fig. 9C). Furthermore, ASF(4M-5R1K) does not alter the final N/C ratio at high ATP levels indicating that phosphorylation regiospecificity within the RS domain is not altered. Taken together, the data suggest that RRM2 contacts affect the extent of processivity, but not the directionality or regiospecificity of RS domain phosphorylation.

Discussion

SRPK1 catalyzes a highly specialized, multi-site phosphorylation reaction that is essential for the localization of SR proteins in the nucleus and subsequent splicing function.³⁰ Prior studies indicate that the serine-specific protein kinase SRPK1 places these phosphates in the RS domain of ASF/SF2 using a regiospecific, directional and processive mechanism.^{23; 24; 25} Recent crystallographic data now illustrate that protein-protein interactions help SRPK1 to

initiate this phosphorylation reaction near the RS1/RS2 boundary (Fig. 1). While these same interactions are likely to support the processive component, it is not clear how static contacts with the RS domain and RRM2 function dynamically to thread the long Arg-Ser chain through the SRPK1 binding channel. We showed previously using ATP-dependent cross-linking experiments that the RS domain moves in the binding channel as a function of phosphorylation.²⁶ These studies show that while the N-terminal portion of RS1 (N'-RS1) is initially bound in the docking groove at the start (Fig.1), this segment moves out of the groove as a function of C-to-N terminal phosphorylation. This process not only requires movement of the RS domain but also induces changes in secondary structure within ASF/SF2. In place of N'-RS1, $\beta 4$ of RRM2 which we label as linker (L), unfolds and moves into the docking groove for the phosphorylation of residues in the N-terminal end of RS1. In this present study, we address how protein-protein interactions including contacts with the RRM2 and the RS domain (Fig. 1B) may accommodate such a 'gliding' RS domain as the reaction progresses from an unphosphorylated to a hyperphosphorylated state.

SRPK1 Uses An Atypical Docking Mechanism for ASF/SF2 Recognition

The SRPK1-ASF/SF2 complex is unusually stable ($K_d \sim 100$ nM) compared to most kinase-substrate pairs. While the present structure is incomplete (Fig. 1), interactions with RRM2 are well-defined and bury nearly 1100 Å² of surface area or about 20% more than that for the N'-RS1 docking groove on SRPK1. These observations suggest that polypeptide regions outside the immediate phosphorylation segment in RS1 may contribute significantly to high affinity. Indeed, many protein kinases use distal contacts far from the immediate phosphorylation segment to enhance binding affinity. For example, the transcription factor substrates for the MAP kinases are not phosphorylated without the presence of amino acids 50-100 residues away from the site of phosphorylation.^{31; 32; 33} Thus, the kinase domain may be presented with several individual binding modules from the protein substrate that cooperatively enhance substrate recognition. This simple model contrasts with that for SRPK1 where a silent, high-affinity binding module far from the local phosphorylation segment in RS1 is not optimally recruited in the initial recognition step. Using a large series of N- and C-terminal deletion constructs we showed that while the RS domain binds as well as the full-length ASF/SF2, the separate RRM1-RRM2 module has the capacity to form a high affinity complex with SRPK1 similar to that for the separate RS domain (Figs. 2 & 5). In fact, the intrinsic high affinity of this RRM1-RRM2 module is likely to be responsible for the unusually stable binding observed in the charge-to-alanine RS domain block mutants and RS2-deleted substrate (Figs. 4 & 5). In all, while ASF/SF2 does not require the RRM1-RRM2 module for initial recognition, these N-terminal domains appear to have an important function when the electropositive character of the RS domain is diminished. This binding feature of SRPK1 is not only very distinct from the classic docking site model, but also appears to be an essential property for sequential, processive phosphorylation of ASF/SF2 as we will discuss next.

Redundant Binding Modules Guide Processivity Through A Strain Mechanism

While the separate RRM1-RRM2 and RS domain modules in ASF/SF2 have high intrinsic binding affinities, the total energies are not realized in the enzyme-substrate complex, at least, prior to phosphorylation. This phenomenon induces some strain within the complex that we believe is important for processive phosphorylation in the presence of building repulsive charge. In our studies we can gauge this strain and its dynamic utilization in start-trap experiments by observing an increase in the level of processivity from about 3 serines in the separate RS domain to 8 serines upon the addition of the RRM1-RRM2 module (Fig. 3). Processivity is achieved in SRPK1 by maintaining a high forward catalytic rate (k_f) and a low substrate dissociation rate (k_{off}). While SRPK1 uses contacts with the RS domain to support the initial processive events (i.e. $-k_f/k_{off} \gg 1$), these contacts are insufficient to support a stable enzyme-substrate complex in later phosphorylation stages due to increased repulsive forces

with the acidic docking groove of SRPK1 (i.e.- $k_f/k_{off} \ll 1$). To keep the enzyme attached for future steps and maintain a high k_f/k_{off} , strain energy stored in the complex is released upon initial RS domain phosphorylation as the RRM1-RRM2 module is recruited for enhanced binding. Although additive modules could also provide stability for initiation, they would not be expected to provide both the stability and flexibility required for later processive steps. For example, if the two binding modules behaved in a purely additive manner, the K_d for the complex would likely be in the sub-picomolar range. This high affinity may fix the substrate too strongly in the active site and may not allow facile movement of the RS domain resulting in a very low k_f . By using an apparently redundant binding module in the form of RRM1-RRM2, SRPK1 balances stability needed for processivity with flexibility needed to allow the requisite structural re-organizations in a lengthy phosphorylation reaction.

Proper Alignment Promotes Efficient Phosphorylation of the RS1 Segment

In addition to catalyzing a processive reaction, SRPK1 also processes the RS domain in a directional (C-to-N-terminal) manner.²⁵ Using the X-ray structure as a guide, we explored whether residue contacts between SRPK1 and RRM2 or the RS1 segment in ASF/SF2 could regulate this ordered phosphorylation mechanism. While mutations in RRM2 that are expected to disrupt interactions with SRPK1 have effects on reaction processivity, they did not impact the directional mechanism (Fig. 9). In contrast, the docking-site defective mutant, SRPK1(6M), not only is a fully distributive kinase (i.e.-nonprocessive) but also displays a greatly relaxed preference for C-to-N-terminal phosphorylation. We believe that this phenomenon is a direct result of a misaligned RS1 segment that can no longer bind dominantly in the manner presented in the X-ray structure (Fig. 1) but instead binds in different initiation modes where N'-RS1 is not consistently engaged in the docking groove at reaction start. A secondary effect of a misaligned RS domain is a greatly reduced rate of ASF/SF2 phosphorylation in single turnover experiments as the enzyme must dissociate the SR protein and re-align for further phosphorylation (Fig. 6). However, while SRPK1(6M) catalyzes a less ordered reaction, the mutations in the docking site do not impact overall phosphoryl content or regiospecificity (Fig. 8), suggesting that other factors control these phenomena. Overall, these findings suggest that the function of the docking groove in SRPK1 is not to increase initial binding affinity as any reduction in charge in the groove could be compensated by the RRM1-RRM2 module but rather is to properly align RS1 in the SRPK1 binding channel.

SRPK1 Uses A Distinctive Pathway For Multi-Site Phosphorylation

Directional phosphorylation has been observed in another protein kinase, GSK-3, where C-to-N-terminal movement is conducted through an electropositive pocket that binds a P+4 phosphoserine.^{28; 34} For GSK-3, phosphorylation of subsequent serines relies on the presence of a C-terminal phospho-serine or 'primed' serine in the consensus sequence -S-X-X-S(P)-. This initial priming step is catalyzed by a secondary kinase as opposed to SRPK1 that catalyzes its own priming step. The detection of a phosphoserine in the SRPK1 structure raises the possibility that an analogous electropositive pocket stabilizing this residue could facilitate the directional mechanism. In such a mechanism, the docking groove would align RS1 for initiation at the C-terminus of RS1 and then the electropositive pocket would help direct subsequent processive steps. Mutations that disrupt the pocket would then be expected to either halt phosphorylation or greatly impair forward progress. In the case of GSK-3 removal of one of the three positively charged residues in the P+4 pocket (i.e.- Arg-96) renders the mutant incapable of phosphorylating a primed substrate.³⁵ In comparison, removal of any or all of the electropositive residues in the P+2 pocket of SRPK1 does not halt phosphorylation of the RS1 segment of ASF/SF2 suggesting that this enzyme differs substantively from GSK-3 in its phosphorylation mode. Unlike the P+4 pocket in GSK-3, the P+2 pocket in SRPK1 does not play a role in the phosphorylation of early sites but instead serves a kinetic role in later steps of the reaction. While the wild-type enzyme readily completes the phosphorylation of the first

10 residues in RS1 in about 90 seconds, the P+2 mutants phosphorylate only half of these residues in this time. Thus, the electropositive pocket in SRPK1 may balance negative charge in the RS domain late in the phosphorylation process.

Model for Processive Phosphorylation

The kinetic data presented here allow us to speculate upon how SRPK1 provides a stable yet flexible platform for the polyphosphorylation of the SR protein ASF/SF2. As shown in Fig. 10, the processive component can be dissected into several events (steps 1-5). While the initial encounter complex is not fully optimal (step 1), it is sufficiently stable to permit the processive phosphorylation of the first three serines (step 2). It is likely that this initial modification then induces a structural change in the complex that allows further optimization of enzyme-substrate interactions (step 3). The nature of this reorganization is not understood but could involve adjustments in the RRM1-RRM2 and RS domain contacts, unfolding of β strand 4 in RRM2 (linker segment) and conformational changes in the kinase domain of SRPK1. As these events are critical for further processivity (step 4), the electropositive P+2 pocket in SRPK1 also helps stabilize the later phosphorylation steps allowing rapid, continued phosphorylation. Despite all these strategies to stabilize the phospho-complex, after about 8 steps, SRPK1 releases the incompletely modified SR protein and then relies on distributive steps to finish RS1 phosphorylation. Interestingly, while we can disrupt processive phosphorylation through RRM1-RRM2 deletion or docking groove mutations, neither alter the overall phosphoryl content of ASF/SF2 raising the question of why SRPK1 evolved such a processive mechanism. Although further studies are needed to address this question, we believe that processivity regulates the biological function of ASF/SF2 by influencing dynamic protein-protein interactions in the context of competing catalytic factors. The sequestration of early phospho-ASF/SF2 intermediates may help promote a unique folded state poised for interaction with nuclear transport proteins and/or may limit the activity of protein phosphatases that would readily lower phosphoryl content and potentially prohibit SR protein shuttling and splicing function.

Materials & Methods

Materials

Lysobacter enzymogenes endoproteinase Lys-C (LysC), adenosine triphosphate (ATP), 3-(*N*-morpholino)propanesulphonic acid (Mops), 2-[*N*-morpholino]ethanesulphonic acid (MES), Tris (hydroxymethyl) aminomethane (Tris), MgCl₂, KCl, NaCl, EDTA, glycerol, Triton X-100 surfactant, acetic acid, Kodak imaging film (Biomax MR), TFA, BSA, acetonitrile, and liquid scintillant were obtained from Fisher Scientific. [γ -³²P] ATP was obtained from NEN Products. α -cyano-4-hydroxy cinnamic acid was obtained from Aldrich Chemicals and recrystallized once from ethanol. Peptides were synthesized from L-amino acids using solid-phase Fmoc chemistry on an Applied Biosystems 433A synthesizer, purified on a C18 HPLC column, and analyzed by electrospray mass spectrometry and absorbance at 215 and 225 nM for concentration determination.

Expression and purification of recombinant proteins

All single and multi-site mutations in ASF/SF2 and SRPK1 were generated by single or sequential polymerase chain reactions using the QuikChange™ mutagenesis kit and relevant primers (Stratagene, La Jolla, CA). C-terminal deletion constructs were made by inserting stop codons in the pET15b (N-terminal His tag) ASF/SF2 construct. N-terminal deletion constructs were generated by PCR amplification and sub-cloning into pET28a (C-terminal His tag). All mutations in SRPK1 were made from a wild-type vector containing an N-terminal His tag (pET15b). Unless otherwise designated, all SRPK1 and ASF/SF2 mutants contain the N-terminal His₆ tag. A kinase inactive form of SRPK1 (kdSRPK1) was generated by replacing

Lys at position 109 with Met and was previously described.³⁶ The synthesis of the cleavage vector, ASF(SR1K), was described previously.²⁵ The plasmids for wild-type and mutant forms of ASF/SF2 and SRPK1 were transformed into the BL21 (DE3) *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 0.4 mM IPTG at room temperature for 5 hours. SRPK1 was purified by Ni-resin affinity chromatography using a published procedure.²³ All ASF/SF2 constructs were refolded and purified using a previously published protocol.²⁴

Phosphorylation Reactions & LysC Proteolysis

The phosphorylation of wild-type and mutant forms of ASF/SF2 by SRPK1 was carried out according to previously published procedures in the presence of 50 mM Mops (pH 7.4), 10 mM free Mg²⁺, 5 mg/mL BSA, and [γ-³²P]ATP (600-1000 cpm pmol⁻¹) at 23 °C.²⁵ Reactions were typically initiated with the addition of ³²P-ATP (100 µM) in a total reaction volume of 10 µL and then were quenched with 10 µL SDS-PAGE loading buffer. Each quenched reaction was loaded onto a 12% SDS-PAGE gel and the dried gels were exposed with Kodak imaging film (Biomax MR). The 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.²³ For equilibrium ATP-dependent phosphorylation mapping (ATP limitation experiments), sample proteolysis with LysC (100 ng) was carried out for 40 min (0.1, 0.2, 0.3, 0.5, 0.75 and 1 µM ATP), 1 h (1.5, 2, and 3 µM ATP) or 4 h (8, and 100 µM ATP) at 37 °C. For kinetic time-dependent phosphorylation mapping (pulse-chase experiments), reactions were initiated with [³²P]ATP (1 or 100 µM) and then were cold-chased at 8s with 100 mM ATP. Proteolysis with LysC (100 ng) was then carried out for 4 h at 37 °C.

Mass Spectrometric Analyses

MALDI-TOF analyses were carried out using a PerSeptive Biosystems Voyager DE PRO spectrometer. Preparation of phosphorylated protein samples was typically carried out using SRPK1 (300 nM) and ASF/SF2 (1 µM) in the presence of 50mM Tris-HCL (pH 7.4), 10mM free Mg²⁺, 1mM DTT at room temperature. Reactions were initiated with the addition of 1 mM ATP in a total volume of 100 µL. Reactions then were quenched with 5% acetic acid, desalted with Zip-tip C₁₈ and eluted with 80% acetonitrile, 2%acetic acid for MALDI-TOF analysis. Unphosphorylated sample controls were prepared in the same manner, without ATP. The matrix solution consisted of 5 mg/ml α-cyano-4-hydroxy cinnamic acid in 1:1:1 acetonitrile, ethanol, 0.52% TFA. Final pH of the matrix solution was 2.0.

Data Analysis

Progress curve data for ASF/SF2 phosphorylation were plotted as ratios of incorporated phosphate and the total substrate concentration (# of sites) as a function of time and were fit to a double-exponential function. Start-trap data were fit to a single exponential function followed by a linear function. The relative initial velocities for the competition data were fit to equation (1):

$$\frac{v_i}{v_o} = \frac{[S] + K_m}{[S] + K_m \left(1 + \frac{[I]}{K_i}\right)} \quad (1)$$

where v_i/v_o is the relative initial velocity (ratio of v in the presence and absence of inhibitor), K_m is the Michaelis constant for the fixed substrate [ASF(ΔRRM1), ASF/SF2 or ASF/SF2-His], $[S]$ is the total concentration of the fixed substrate, $[I]$ is the total concentration of the

competitive inhibitor and K_I is the dissociation constant for the inhibitor. The relative initial velocities for the partial competitive data were fit to equation (2):²⁹

$$\frac{v_i}{v_o} = \frac{[S] + K_m}{[S] + K_m \left[\frac{1 + [I]/K_I}{1 + [I]/\alpha K_I} \right]} \quad (2)$$

where v_i/v_o , K_m , $[S]$, and $[I]$ are the same as in equation (1) and K_I and αK_I are the dissociation constants for the partial competitive inhibitor in the absence and presence of S (see Scheme 1).

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Figure 1. Structural features of the SRPK1:ASF/SF2 Complex

(A) ASF/SF2 domain organization and C-terminal sequences. The RS domain is composed of RS1 and RS2 segments where the former is phosphorylated by SRPK1. L is a linker segment separating the RS domain from the canonical RRM. (B) X-ray structure of SRPK1 in complex with a truncated form of ASF/SF2 bound.²⁶ SRPK1 lacking the insert domain is shown with an electrostatic surface where red represents negative charge and blue represents positive charge. The truncated ASF/SF2 lacks RRM1 and residues 221-248. A portion of RS1 (N'-RS1) is visible in the binary complex whereas the preceding sequence (PRSP) and residues 211-219 are disordered. The linker segment is the final β strand of RRM2. A dipeptide containing a

phosphoserine that interacts with the P+2 pocket in SRPK1 comes from one of the serines in the C-terminal portion of RS1.

Figure 2. Effects of N-terminal deletions on the binding of ASF/SF2

(A) Deletion Mutants. All deletion constructs contain a C-terminal His₆ tag. (B) Competition assay. Phosphorylation of ASF/SF2-His (50 nM) is monitored as a function of varying concentrations of competitor substrates (0-1600 nM) using 100 μM ³²P-ATP. ASF(ΔRRM1)-His is the fixed substrate when the competitor is ASF/SF2-His. Relative velocities are plotted against total ASF/SF2-His (●), ASF(ΔRRM1)-His (○), ASF(ΔRRM12)-His (▲), and ASF(RS12)-His (△) concentrations and fitted to equation (1) to obtain true K_I values of 93 ± 14, 54 ± 5, 70 ± 15, and 190 ± 13 nM, respectively. (C) Double reciprocal plot of initial velocity versus varying ASF/SF2-His concentration using 0 nM (●), 100 nM (■) and 200 nM (▲) ASF(ΔRRM12)-His, respectively. (D) MALDI-TOF spectra of ASF/SF2-His and ASF(ΔRRM12)-His with SRPK1 in the absence and presence of 100 μM ATP. The increase in mass is consistent with the phosphorylation of about 14 sites for ASF/SF2-His and ASF(ΔRRM12)-His.

Figure 3. Kinetic investigation of ASF/SF2 lacking one or both RRMs

(A) Single turnover progress curves. SRPK1 (1 μ M) is incubated with 250 nM of ASF/SF2 (●), ASF(Δ RRM1) (○), ASF(Δ RRM2) (▲) and ASF(Δ RRM12) (▲) and the reaction is initiated with the addition of 100 μ M 32 P-ATP. The data are fit to a double exponential equation with rate constants of 3.7 and 0.13 min^{-1} and amplitudes of 12 and 2 phosphates for ASF/SF2, rate constants of >10 and 0.11 min^{-1} and amplitudes of 10 and 4 phosphates for ASF(Δ RRM1), rate constants of >10 and 0.15 min^{-1} and amplitudes of 12 and 2 phosphates for ASF(Δ RRM2), and rate constants of >10 and 0.12 min^{-1} and amplitudes of 10 and 4 phosphates for ASF(Δ RRM12). (B-E) Start-trap experiments. SRPK1 (1 μ M) is pre-equilibrated with 250 nM of ASF/SF2 (B), ASF(Δ RRM1) (C), ASF(Δ RRM2) (D), and ASF(Δ RRM12) (E) and then allowed to react with 100 μ M 32 P-ATP in the absence (○) and presence (start-trap) (●) of 30 μ M kdSRPK1 added at the time of reaction start. In control experiments (trap-start), kdSRPK1 is added prior to reaction start (▲). Start-trap data are fit to single exponentials followed by linear functions. Amplitudes are lowered from 12 to 8.1 sites for ASF/SF2, from 10 to 6.1 sites for ASF(Δ RRM1), from 12 to 6.9 sites for ASF(Δ RRM2) and from 9.7 to 2.8 sites for ASF(Δ RRM12) in the absence and presence of kdSRPK1. Trap-start experiments are fit to linear functions with slopes of 1, 1.6, 3.0 and 3.5 sites/min for ASF/SF2, ASF(Δ RRM1), ASF(Δ RRM2) and ASF(Δ RRM12), respectively. (F) Effects of deletion on processivity. Amplitudes (# sites) in start-trap experiments in (B-E) are plotted in the bar graph.

Figure 4. Effects of RS domain charge on ASF/SF2 binding

(A) Mutant and peptide substrates. Charge-to-Ala block mutations are made in the RS domain of ASF/SF2. Peptide substrates contain 8 and 16 Arg-Ser repeats. (B) Relative velocities for the phosphorylation of ASF(Δ RRM1) (50 nM) are plotted as a function of varying wt-ASF/SF2 ($\square$), Block1 ($\bullet$), Block 2 ($\bigcirc$), Block 3 ($\blacktriangle$), Block 4 ($\triangle$) and fit to equation (1) to obtain K_I values of 90 ± 11 , 130 ± 20 , 220 ± 20 , 190 ± 20 , and 73 ± 10 , respectively. (C) Relative initial velocities for the phosphorylation of ASF(Δ RRM1) (50 nM) are plotted as a function of varying Block 1/2 ($\bigcirc$), Block 2/3 ($\bullet$), and Block 3/4 ($\triangle$) and fit to equation (2) to obtain K_I values of 40 ± 30 , 45 ± 30 , and 120 ± 30 nM, respectively, and αK_I values of 60 ± 40 , 100 ± 40 , and 800 ± 360 , respectively. (D) Relative velocities for the phosphorylation of ASF/SF2

(50 nM) are plotted as a function of varying (RS)₈ (●) and (RS)₁₆ (○) and fit to equation (1) to obtain K_I values of 1.8 ± 0.22 and 0.16 ± 0.041 μM.

Figure 5. C-terminal deletion analyses of ASF/SF2

(A) Deletion constructs. (B) Competition assay. Phosphorylation of ASF/SF2 (50 nM) is monitored as a function of varying concentrations of competitor substrates (0-16 μ M) using 100 μ M 32 P-ATP. Relative initial velocities are plotted against total ASF(Δ RS2) (○), ASF(Δ RS) (●), ASF(4M- Δ RS) (□), ASF(RRM1) (▲), and ASF(RRM2) (▲) and fit to true K_I values of 170 ± 60 , 180 ± 20 , 200 ± 30 , 1600 ± 400 and 6300 ± 1900 nM, respectively. The dotted line shows relative initial velocities for ASF/SF2 taken from Fig 4B. (C) Double reciprocal plot of initial velocity versus varying ASF/SF2 concentration using 0 nM (●), 400 nM (▲) and 800 nM (■) ASF(Δ RS), respectively. (D) Start-trap experiments. SRPK1 (1 μ M) is pre-equilibrated with 250 nM of ASF(Δ RS2) and then allowed to react with 100 μ M 32 P-ATP in the absence (○) and presence (start-trap) (●) of 30 μ M kdSRPK1 added at the time of reaction start. In control experiments (trap-start), kdSRPK1 is prior to reaction start (▲) and fit to a linear function with a slope of 1.3 sites/min.

Figure 6. Effects of docking groove mutations on phosphorylation kinetics

(A) Docking groove in SRPK1. Residues from N'-RS1 are shown in gold. (B) Single turnover kinetics. SRPK1(6M) (3 μ M) is pre-equilibrated with ASF/SF2 (250 nM) and then mixed with 100 μ M 32 P-ATP. The curve is fit to a double exponential function with rate constants and amplitudes of 1 min^{-1} and 5.5 sites and 0.07 min^{-1} and 9.5 sites. Kinetic data for wild-type SRPK1 are taken from Fig. 3A and are displayed as a dotted line. (C) Binding affinity. Competition experiments are performed using 50 nM ASF(Δ RRM2), varying amounts of ASF/SF2 and 15 nM SRPK1(6M). A true K_I of 240 ± 50 nM for ASF/SF2 to SRPK1(6M) is obtained using equation (1). The dotted line shows the inhibitor plot for wild-type SRPK1 and ASF/SF2 taken from Fig. 4B. (D) Start-Trap Experiment. SRPK1(6M) (3 μ M) and ASF/SF2 (250 nM) are pre-equilibrated before 100 μ M 32 P-ATP is added in the absence (○) and presence (●) of 60 μ M kdSRPK1 at the time of start. In the trap-start experiments, kdSRPK1 is added to the enzyme-substrate complex before addition of 32 P-ATP. The amplitude is lowered from 5.6 sites in the absence to 1.2 sites in the presence of kdSRPK1. The slope of the trap-start experiment is 0.2 sites/min.

Figure 7. Role of the electropositive pocket in late phosphorylation steps

(A) Electropositive pocket. Phospho-serine (P-Ser) interacts with three positively charged residues from the P+1 loop and helix α G. (B) Binding affinities. Competition experiments are performed using 50 nM ASF(Δ RRM2), varying amounts of ASF/SF2 and 15 nM of each enzyme. True K_I values of 150 ± 23 , 110 ± 21 , 240 ± 36 , and 220 ± 15 nM were obtained for wt-SRPK1 (Δ), SRPK1(RA) ($\blacktriangle$), SRPK1(2RA) ($\bigcirc$), SRPK1(3RA) ($\bullet$), respectively. K_m values for ASF(Δ RRM2) of 95, 110, 139 and 134 nM were obtained for wt-SRPK1, SRPK1(RA), SRPK1(2RA), SRPK1(3RA), respectively, and used for determining the true K_I 's. (C) Single turnover kinetics. Enzymes (2 μ M) were preequilibrated with ASF/SF2 (250 nM) and reacted with 100 μ M 32 P-ATP. The data are fit to double exponential functions with initial amplitudes of 10.5, 5.5, 7.1, and 5.6 sites for wt-SRPK1, SRPK1(RA), SRPK1(2RA), SRPK1(3RA), respectively. (D) Start-trap kinetics. SRPK1(3RA) (2 μ M) is preequilibrated with ASF/SF2 (250 nM) before 100 μ M 32 P-ATP is added in the absence ($\bigcirc$) and presence ($\bullet$) of 60 μ M kdSRPK1 at the time of start. In the trap-start experiments, kdSRPK1 is added to the enzyme-substrate complex before addition of 32 P-ATP. The slope of the trap-start data is 0.2 sites/min.

Figure 8. Docking groove mutants in SRPK1 affect directional phosphorylation of ASF/SF2
 (A) Digestion fragmentation of ASF/SF2. Mutant form of ASF/SF2 [ASF(5R1K)] with a single lysine mutations in the center of RS1 (Arg-214) and several Lys-to-Arg mutations in RRM2 can be cleaved by LysC into two phosphorylated fragments. Solid arrow heads indicate the locations of LysC cleavage sites. (B) ATP limitation assays. ASF(5R1K) (250 nM) is preequilibrated with 3 μM wt-SRPK1, SRPK1(3RA) and SRPK1(6M) before the addition of varying amounts of ³²P-ATP. N- and C-terminal fragments upon LysC treatment are then resolved by SDS-PAGE autoradiography. (C) Fragmentation pattern as a function of phosphoryl content. The ratios of N- and C-terminal proteolytic fragments are plotted as a function of the total phosphoryl content of ASF(5R1K). The total phosphoryl content is measured from reactions not treated with LysC. (D) Pulse-chase experiments. SRPK1:ASF (5R1K) complexes are mixed with 1 or 100 μM ³²P-ATP (pulse) and allowed to react for 8 seconds before adding 10 mM cold ATP (chase). N- and C-terminal fragments are then generated with LysC.

Figure 9. Effects of RRM2 mutations on phosphorylation kinetics and directionality

A) Single turnover kinetics. SRPK1 (1 μM) is incubated with 250 nM of ASF(4M) and the reaction is initiated with the addition of 100 μM ^{32}P -ATP. The data are fit to a double exponential equation with rate constants of >18 and 0.57 min^{-1} and amplitudes of 8.7 and 5.3 phosphates. The dotted line is the single turnover kinetics for ASF/SF2 taken from Fig. 3A. (B) Start-trap experiment. SRPK1 (1 μM) is pre-equilibrated with 250 nM of ASF(4M) and then allowed to react with 100 μM ^{32}P -ATP in the absence (○) and presence (●) of 60 μM kdSRPK1 added at the time of reaction start. In trap-start experiments, kdSRPK1 is added prior to reaction start (▲) and fit to a linear function with a slope of 2.3 sites/min. The start-trap data are fit with a single exponential with a rate constant of $>20 \text{ min}^{-1}$ and amplitude of 5.4 sites followed by a linear function of 2.3 sites/min. (C) ATP limitation experiments. ASF (4M-5R1K) (250 nM) is preequilibrated with 3 μM wt-SRPK1 before the addition of varying amounts of ^{32}P -ATP. N- and C-terminal fragments upon LysC treatment are then resolved by SDS-PAGE autoradiography and the ratio of N- and C-terminal proteolytic fragments are plotted as a function of total phosphoryl content of ASF(5R1K). The total phosphoryl content is measured from reactions not treated with LysC.

Figure 10. Model for processive phosphorylation of ASF/SF2

SRPK1 (blue) contains a binding channel for the RS domain (black line), the P+2 electropositive pocket, and binding surfaces for RRM1 and RRM2 (both in red). The processive cycle includes 5 steps that involve substrate recognition (1), initial processive phosphorylation of ~3 serines (2), rearrangement step to optimize contacts (3), continuation of processive phosphorylation of ~5 serines (4), and release of phospho-ASF/SF2. Phosphates are shown in green.

Scheme 1.