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Splicing Kinase SRPK1 Conforms to the Landscape of Its SR Protein Substrate

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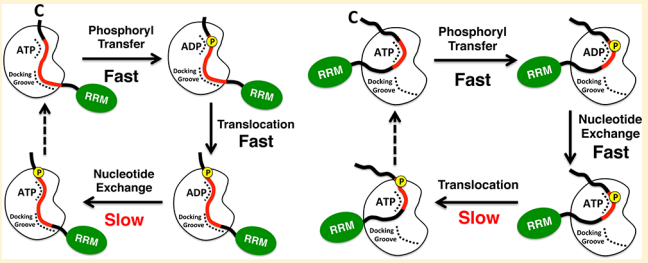
Supporting Information

ABSTRACT: The splicing function of SR proteins is regulated by multisite phosphorylation of their C-terminal RS (arginine−serine rich) domains. SRPK1 has been shown to phosphorylate the prototype SR protein SRSF1 using a directional mechanism in which 11 serines flanked by arginines are sequentially fed from a docking groove in the large lobe of the kinase domain to the active site. Although this process is expected to operate on lengthy arginine−serine repeats (≥8), many SR proteins contain smaller repeats of only 1−4 dipeptides, raising the question of how alternate RS domain configurations are phosphorylated. To address this, we studied a splice variant of Tra2β that contains a C-terminal RS domain with short arginine−serine repeats [Tra2β(ΔN)]. We showed that SRPK1 selectively phosphorylates several serines near the C-terminus of the RS domain. SRPK1 uses a distributive mechanism for Tra2β(ΔN) where the rate-limiting step is the dissociation of the protein substrate rather than nucleotide exchange as in the case of SRSF1. Although a functioning docking groove is required for efficient SRSF1 phosphorylation, this conserved structural element is dispensable for Tra2β(ΔN) phosphorylation. These large shifts in mechanism are likely to account for the slower net turnover rate of Tra2β(ΔN) compared to SRSF1 and may signal fundamental differences in phosphorylation among SR proteins with distinctive arginine−serine profiles. Overall, these data indicate that SRPK1 conforms to changes in RS domain architecture using a flexible kinetic mechanism and selective usage of a conserved docking groove.



The splicing of precursor mRNA (pre-mRNA) occurs in a macromolecular complex composed of several small nuclear RNAs and more than 100 auxiliary protein factors.[1] This complex, known as the spliceosome, establishes the correct 5′−3′ splice sites and catalyzes the necessary trans-esterification reactions for splicing. Many proteins involved in this process contain polypeptide regions enriched in Arg−Ser dipeptide repeats known as RS domains. Most notably, the SR proteins are an essential family of splicing factors that derive their names from the presence of C-terminal RS domains. SR proteins typically bind to exonic sequences in pre-mRNA via their RNA recognition motifs (RRMs), recruiting essential elements of the spliceosome such as U1 snRNP at the 5′ splice site and U2AF65 at the 3′ splice site.[2,3] The activities of SR proteins are regulated through RS domain phosphorylation. The SRPK family of serine kinases phosphorylates SR proteins in the cytoplasm, a modification that initiates contacts with a transportin protein and directs the splicing factor into the nucleus.[4,5] SR proteins can undergo additional phosphorylation in the nucleus by SRPKs and the CLK family of protein kinases.[6] There is now strong data supporting the notion that RS domain phosphorylation by these two kinase families not only controls the subcellular localization of SR proteins but also their role in gene splicing.[7−11] How SR protein phosphorylation controls splicing is not fully understood, but recent progress suggests that the RS domain may regulate RRM interactions with pre-mRNA in a phosphorylation-dependent manner that requires the concerted activities of SRPKs and CLKs.[12]

The RS domains in SR proteins can range from only 50 to over 300 residues in length, and the Arg−Ser dipeptide repeats can vary in both length and position. To date, there is no universal understanding of how these domains are modified by SRPKs and CLKs or how specific phosphorylation regulates SR protein activities in splicing. Much of what we know about SR protein phosphorylation has been garnered from studies on the SR protein SRSF1 (aka ASF/SF2). SRSF1 is considered the prototype for the SR protein family and is the best understood to date. It possesses two RRMs (RRM1 and RRM2) and a short RS domain (Figure 1A). Prior kinetic studies showed that SRPK1 rapidly phosphorylates a long Arg−Ser stretch using a semiprocessive, sequential mechanism in which the kinase binds with high affinity to the C-terminal end of the repeat and adds phosphates in a strict N-terminal direction.[13,14] This directional pathway is enforced by an electronegative docking groove in the large lobe of the kinase domain that systematically feeds N-terminal Arg−Ser dipeptides into the active site of SRPK1.[15] Translocation of the Arg−Ser dipeptides is highly efficient such that ADP release limits the addition of

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each phosphate.[16,17] Once this reaction is complete, SRPK1 can migrate to the C-terminal end of the RS domain and then slowly phosphorylate a shorter Arg−Ser stretch (three repeats). CLK1 can modify the same serines as SRPK1, albeit at a slower rate. The kinase can also phosphorylate serines that flank prolines, an activity unique to the CLK family.[18,19] Unlike SRPK1, which moves in a highly directional manner, CLK1 appears to randomly phosphorylate the RS domain of SRSF1.[20] Despite these differences, both kinases bind SRSF1 with high affinity (K_d < 100 nM).[21]

Although SRSF1 contains a lengthy Arg−Ser repeat region (Figure 1A), many SR proteins contain smaller repeats of only 1−4 dipeptides that are unevenly and widely distributed in the RS domains (Figure S1). Whether SRPK1 uses a common mechanism to modify these shorter repeats is not understood at this time. To investigate the diverse modes of RS domain phosphorylation, we chose an alternative substrate that contains shorter Arg−Ser repeats in its C-terminal RS domain. Tra2β1 is an SR-like protein that contains two RS domains that flank the N- and C-terminal ends of a central RRM and is known to regulate the splicing of several genes, including survival motor neuron 2 and Tau, in a phosphorylation-dependent manner.[22,23] In addition to controlling pre-mRNA binding, RS domain phosphorylation blocks inclusion of exons 2 and 3, resulting in the expression of a truncated protein form of Tra2β1 (Tra2β3) that lacks the N-terminal RS domain and thus possesses the classic domain organization of an SR protein.[24] This splicing switch has been correlated with the generation of Tau isoforms that could be linked to neurodegenerative diseases.[25] The C-terminal RS domain of Tra2β3 differs from that of SRSF1 in that it lacks a lengthy Arg−Ser repeat and thus serves as a useful substrate for exploring the mechanism of SRPK1 toward alternate RS domains. We expressed and studied the phosphorylation of a form of Tra2β1 that lacks the N-terminal RS domain (Tra2β(ΔN)), mimicking the splice variant Tra2β3. We found that Tra2β(ΔN) is rapidly phosphorylated in the active site of SRPK1 but is turned over very slowly compared to the prototype SRSF1. This is due to a distributive phosphorylation mechanism in which protein translocation/release limits the net turnover. These mechanistic changes account for the sluggish activation of Tra2β(ΔN) compared to SRSF1 and may reflect a general phenomenon for SR proteins with shorter Arg−Ser repeats. As a result of the changes in the structure of the RS domain, the interaction of SRPK1 with Tra2β(ΔN) is profoundly different than that with SRSF1. Mutagenesis experiments show that a conserved docking groove in SRPK1, critical for rapid, directional phosphorylation of SRSF1, is dispensable for Tra2β(ΔN) phosphorylation. These results imply that splicing kinases such as SRPK1, adapt to changes in the structural landscape of its substrate using unique mechanisms of catalysis tailored to the nature of the RS domain.

MATERIALS AND METHODS

Materials. Adenosine triphosphate (ATP), 3-(*N*-morpholino)propanesulphonic acid (Mops), Tris-(hydroxymethyl) aminomethane (Tris), $MgCl_2$, NaCl, EDTA, glycerol, sucrose, acetic acid, lysozyme, DNase, RNase, Phenix imaging film, BSA, Whatman P81 grade filter paper, and liquid scintillant were obtained from Fisher Scientific. *Lysobacter enzymogenes* endoproteinase Lys-C (LysC) and protease inhibitor cocktail were obtained from Roche. [γ-^{32}P]ATP was obtained from NEN Products, a division of PerkinElmer Life Sciences.

Expression and Purification of Recombinant Proteins. SRPK1, CLK1, and SRSF1 were expressed from a pET19b vector containing a 10× His Tag at the N terminus.[19] Tra2β(ΔN), which lacks resides 1−113 from Tra2β1, was expressed from pET28a containing a C-terminal 10× His Tag. All mutations in Tra2β(ΔN) were generated by single or sequential polymerase chain reactions using the QuikChange mutagenesis kit and relevant primers (Stratagene, La Jolla, CA). All deletion constructs of Tra2β(ΔN) were generated using the Thermo Scientific Phusion Site-Directed Mutagenesis Kit and relevant primers (Integrated DNA Technologies, San Diego, CA). The plasmids for wild-type and mutant forms of Tra2β(ΔN), SRPK1, SRSF1, and CLK1 were transformed into the BL21 (DE3) *Escherichia coli* strain and grown at 37 °C in LB broth supplemented with 100 μg/mL of ampicillin. Protein expression was induced with 1 μg/mL of IPTG at room temperature for 5 h for SRSF1 and Tra2β(ΔN) and for 12 h for SRPK1 and with 2.5 μg/mL of IPTG for 16 h for CLK1. SRPK1, SRPK1(6M), CLK1, and Tra2β(ΔN/RS) were purified by Ni-resin affinity chromatography using published procedures.[21,26] All SRSF1 and Tra2β(ΔN) constructs containing part or all of the RS domain were refolded and purified using a previously published protocol.[13]

Phosphorylation Reactions (Manual Mixing). The phosphorylation of wild-type and mutant forms of Tra2β(ΔN) and SRSF1 by SRPK1 and CLK1 were carried out in the presence of 100 mM Mops (pH 7.4), 10 mM free Mg^{2+}, and 5 mg/mL of BSA at 23 °C according to previously published procedures.[13] Progress curves were carried out with 1 μM enzyme and 0.2 μM SR protein and 100 μM [γ-^{32}P]ATP (4000−8000 cpm pmol^{-1}) unless otherwise stated. Competition reactions were carried out using fixed amounts of Tra2β(ΔC) (500 nM) or Tra2β(ΔN) (1 μM) as substrates and varying concentrations of the competitors (substrate inhibitors). All reactions were carried out in a total reaction volume of 10 μL and were then quenched with 10 μL of SDS-PAGE loading buffer. Phosphorylated SR protein was separated from unreacted [γ-^{32}P]ATP by loading the quenched reaction on an SDS-PAGE gel (10 or 16%) and running at 170 V for 1 h. Protein bands corresponding to phosphorylated SR protein were cut from the dried SDS-PAGE gel and quantitated on the ^{32}P channel in liquid scintillant. The total amount of phosphoproduct was then determined by considering the specific activity (cpm min^{-1}) of the reaction mixture and the background retention of [γ-^{32}P]ATP in the absence of enzyme.

LysC Proteolysis and Pull Downs. Tra2β(ΔN) (0.25 μM) was phosphorylated using 1 μM SRPK1 in the presence of 50 μM [γ-^{32}P]ATP (4000−8000 cpm pmol^{-1}) for 30 min in a total volume of 70 μL. This reaction was then split in half, and an equal volume of buffer (50 mM Tris pH 8.5, 2 mM EDTA) with and without 0.35 μg of LysC was added and incubated at 37 °C for 90 min. For pull-down assays, portions of these reactions were incubated with and without 30 μL of Ni^{2+} resin at room temperature for 60 min on a benchtop rotator. The incubated reactions containing Ni^{2+} resin were then washed six times with 500 μL of buffer (25 mM Tris pH 8.5, 0.5% Triton-X 100, 1 M NaCl, 15% Glycrol). SDS-PAGE loading buffer was added to the samples, which were then boiled for 5 min to release the protein fragments bound to the Ni^{2+} resin before spinning down for 5 min at 12 000 rpm. Phosphorylated SR

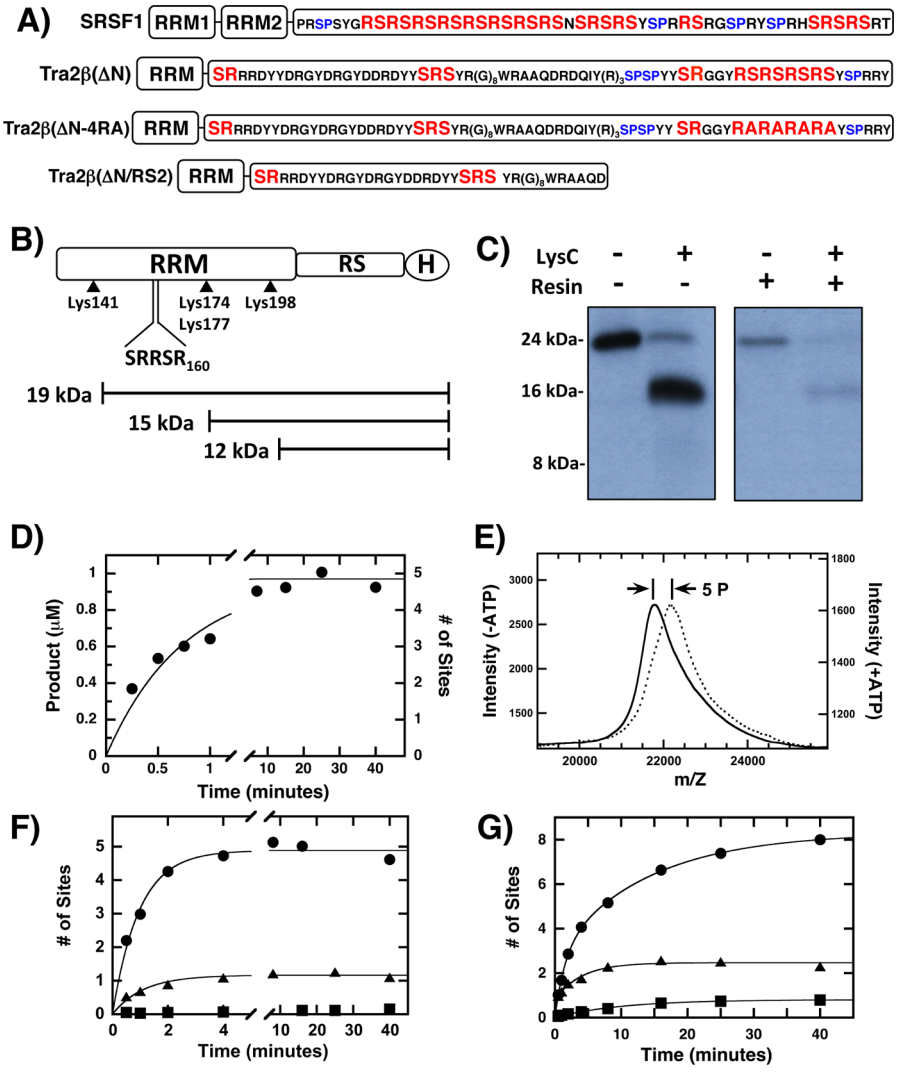

Figure 1. Mapping phosphorylation sites in Tra2β(ΔN). (A) C-terminal RS domain sequences in SRSF1, Tra2β(ΔN), Tra2β(ΔN-4RA), and Tra2β(ΔN/RS2). Ser−Pro and Arg−Ser dipeptides are colored blue and red, respectively. (B) Lysines in Tra2β(ΔN). Expected LysC fragment sizes containing the C-terminal His tag (H). (C) LysC cleavage of Tra2β(ΔN). The substrate is phosphorylated using SRPK1 and [γ-^{32}P]ATP and proteolyzed with LysC. The products are bound to the Ni resin and washed. (D) Progress curve for the phosphorylation of Tra2β(ΔN) (0.2 μM) by SRPK1 (1 μM). The data are fit to a single exponential function with an amplitude of 0.97 ± 0.04 μM (4.8 sites) and a rate constant of 1.4 ± 0.16 min^{-1}. (E) MALDI−TOF for Tra2β(ΔN). The major peak at 21.7 kDa for unphosphorylated Tra2β(ΔN) (solid line) increases to 22.1 kDa in the presence of ATP (dotted line), consistent with the addition of five phosphates. (F) Progress curves for the phosphorylation of Tra2β(ΔN) (●), Tra2β(ΔN-4RA) (▲), and Tra2β(ΔN/RS2) (■) by SRPK1. The data were fit to amplitudes of 4.9 ± 0.1 and 1.2 ± 0.05 sites and rate constants of 1 ± 0.1 and 0.9 ± 0.14 min^{-1} for Tra2β(ΔN) and Tra2β(ΔN-4RA). (G) Progress curves for the phosphorylation of Tra2β(ΔN) (●), Tra2β(ΔN-4RA) (▲), and Tra2β(ΔN/RS2) (■) by CLK1. The data were fit to a double exponential function with amplitudes of 2.9 ± 0.26 and 5.4 ± 0.19 sites and rate constants of 0.65 ± 0.07 and 0.07 ± 0.007 min^{-1} for Tra2β(ΔN) and amplitudes of 0.8 ± 0.23 and 1.7 ± 0.07 sites and rate constants of 5.9 ± 3 and 0.25 ± 0.07 min^{-1} for Tra2β(ΔN-4RA). The data for Tra2β(ΔN/RS2) were fit to a single exponential function with an amplitude and rate constant of 0.8 ± 0.02 sites and 0.10 ± 0.01 min^{-1}.

protein fragments were visualized using a 20% SDS-PAGE gel run at 170 V for 1 h.

Mass Spectrometric Analyses. MALDI−TOF analyses were carried out using a Voyager DE-STR spectrometer. Tra2β(ΔN) (1 μM) was incubated with SRPK1 (300 nM) and 0.3 mM ATP in the presence of 50 mM Mops (pH 7.4) and 10 mM free Mg^{2+} for 1 h in a total volume of 100 μL at room temperature. Reactions then were quenched with 5% acetic acid, desalted with Zip-tip C_4, and eluted with 80% acetonitrile and 2% acetic acid for MALDI−TOF analysis. Unphosphorylated sample controls were prepared in the same manner without ATP. The matrix solution consisted of sinapinic in 70% methanol and 0.05% TFA. Final pH of the matrix solution was 2.0.

Rapid Quench-Flow Experiments. Pre-steady-state and single-turnover kinetic measurements were performed using a KinTek Corp. model RGF-3 quench-flow apparatus following a previously published procedure.[17] The apparatus consists of three syringes driven by a stepping motor. Typical experiments were performed by mixing equal volumes of the SRPK1−Tra2β(ΔN) complex in one reaction loop and [γ-^{32}P]ATP (5000−15000 cpm pmol^{-1}) in the second reaction loop in the presence of 100 mM Mops (pH 7.4), 10 mM free Mg^{2+}, and 5 mg/mL of BSA. The reaction was quenched with 30% acetic acid in the third syringe. Phosphorylated Tra2β(ΔN) was

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separated from unreacted ATP using a filter-binding assay where a portion of each quenched reaction (50 μL) was spotted onto a phosphocellulose filter disk and was washed three times with 0.5% phosphoric acid. The filter disks were rinsed with acetone, dried, and counted on the ^{32}P channel in liquid scintillant. The total amount of phosphoproduct was then determined by considering the specific activity (cpm min^{-1}) of the reaction mixture, and the background retention of [γ-^{32}P]ATP in the absence of enzyme. Retention of the phosphorylated product on the filters was assessed by running quenched reaction samples on SDS-PAGE gels and counting the bands. Control experiments lacking the Tra2β(ΔN)−SRPK1 complex were run to define a background correction.

Viscosity Studies. The steady-state phosphorylation of Tra2β(ΔN) was monitored using the filter-binding assay as described above in the presence of 0−30% sucrose. The relative solvent viscosity (η^{rel}) of the buffer (100 mM Mops, pH 7.4) containing 0−30% sucrose was measured using an Ostwald viscometer and a previously published protocol.[27] A η^{rel} value of 1.44, 1.83, 2.32, and 3.43 was measured for buffer containing 10, 20, 25, and 30% sucrose at 23 °C.

Data Analysis. In single-turnover experiments, the time-dependent production of phosphoproduct was fit to either single or double exponential functions. In pre-steady-state kinetic experiments, the reaction product normalized to the enzyme concentration ([P]/[E]) were fit to eq 1

$$\frac{[P]}{[E]} = \alpha(1 - \exp(-k_b t)) + k_L t \quad (1)$$

where α, k_b, and k_L are the amplitude of the 'burst' phase, the rate constant for the burst phase, and the rate constant for the linear phase, respectively. The initial velocity data were fit to the Michaelis−Menten equation to obtain K_m and V_{max}. The V_{max} values were converted to k_{cat} using the total enzyme concentration determined from a Bradford assay ($k_{cat} = V_{max}/E_{tot}$). The rate constants for several steps in the mechanism were extracted from viscosity dependences on several steady-state kinetic parameters according to eqs 2−5

$$k_4 = \frac{k_{cat}}{(k_{cat})^{\eta}} \quad (2)$$

$$k_3 = \frac{k_{cat}}{[1 - (k_{cat})^{\eta}]} \quad (3)$$

$$k_{on}^{ATP} = \frac{k_{cat}/K_{ATP}}{(k_{cat}/K_{ATP})^{\eta}} \quad (4)$$

$$k_{off}^{ATP} = \left(\frac{k_{cat}}{(k_{cat}/K_{ATP})^{\eta}}\right)\left(\frac{1 - (k_{cat}/K_{ATP})^{\eta}}{1 - (k_{cat})^{\eta}}\right) \quad (5)$$

where $(k_{cat})^{\eta}$ and $(k_{cat}/K_{ATP})^{\eta}$ are the slopes of plots of the relative steady-state kinetic parameter versus η^{rel}. The dissociation constant (K_I) for a protein construct from SRPK1 was measured using eq 6

$$\frac{v}{v_o} = \frac{[S] + K_{SR}}{[S] + K_{SR}\left(1 + \frac{[I]}{K_I}\right)} \quad (6)$$

where v_i/v_o is the relative initial velocity (ratio of v in the presence and absence of inhibitor) and [I] is the total substrate inhibitor concentration.

RESULTS

SRPK1 Phosphorylates the RS domain in Tra2β(ΔN). Because SRPK1 has been shown to phosphorylate exclusively the RS domain of SRSF1[14] (Figure 1A), we wondered whether other SR proteins show similar regiospecificity. To address this question, we expressed and purified a natural splice variant of the SR protein, Tra2β1, that lacks the N-terminal RS domain. This form, named Tra2β(ΔN) throughout, retains the traditional structure of an SR protein with an RRM followed by a C-terminal RS domain (Figure 1A). Although Tra2β(ΔN) contains eight serines flanking arginines in its RS domain, the single RRM also contains potential SRPK1 sites (SRRSR$_{160}$; Figure 1B). To identify whether SRPK1 targets the latter serines, we performed a footprinting experiment. Tra2β(ΔN) contains four lysines in the RRM (none in the RS domain) that may be cleaved by the protease LysC (Figure 1B). Although Lys-141 lies on the N-terminal side of the potential SRPK1 sites, the remaining lysines (Lys-174, -177, and -198) lie on the C-terminal side, and their cleavage can be used to map phosphorylation in the putative SRPK1 sequence. Upon phosphorylation and LysC cleavage, Tra2β(ΔN), which typically migrates as a 24 kDa protein by SDS-PAGE, reduces to a 16 kDa polypeptide (Figure 1C). The radioactivity in this fragment and the remaining substrate account for most (95%) of the total, indicating that LysC generates only one major phosphorylated fragment from Tra2β(ΔN). Also, we observed no small bands on the autoradiogram, consistent with a fragment spanning the putative SRPK1 site in the RRM. Pull-down experiments verify that the 16 kDa fragment contains the original C-terminal His tag (Figure 1B) and likely reflects cleavage at Lys-174 or Lys-177 rather than Lys-141, which is expected to produce a 19 kDa polypeptide. Because Lys-174/177 are C-terminal to the putative phosphorylation segment, SRPK1 is unlikely to phosphorylate within SRRSR$_{160}$. To confirm these results, we expressed the RRM of Tra2β(ΔN) and found that it was not phosphorylated by SRPK1 (data not shown). These results indicate that SRPK1 specifically phosphorylates the C-terminal RS domain in Tra2β(ΔN).

Identification of Phosphorylation Sites in the RS domain. To identify which of the serines in the Tra2β(ΔN) RS domain is modified by SRPK1, we initially performed kinetic experiments to measure the total phosphoryl content. Although the RS domain contains eight serines flanking arginines, approximately five of those serines are modified by SRPK1 within the time frame of the assay (Figure 1D). This total phosphoryl content obtained from the autoradiogram was verified using MALDI−TOF mass spectrometry (Figure 1E). To address whether the final Arg−Ser repeat in the RS domain ((RS)$_4$) corresponds to the major sites, we expressed and purified a serine-to-alanine mutant that removes these four serines (Tra2β(ΔN-4RA)) and showed that it is poorly phosphorylated by SRPK1 (Figure 1A,F). The reduction in observed phosphoryl content (5 → 1) suggests that SRPK1 mainly targets the final (RS)$_4$ region and may also modify one additional isolated Arg−Ser dipeptide. To determine which serines are modified, we designed a deletion mutant that removes (RS)$_4$ and one additional nearby serine. Tra2β(ΔN/RS2) is not a substrate for SRPK1 (Figure 1F), indicating that the Ser directly N terminal to (RS)$_4$ is phosphorylated along with (RS)$_4$. Furthermore, the inability to phosphorylate Tra2β(ΔN/RS2) supports the observation that SRPK1 does not phosphorylate within the RRM. To address whether the C-

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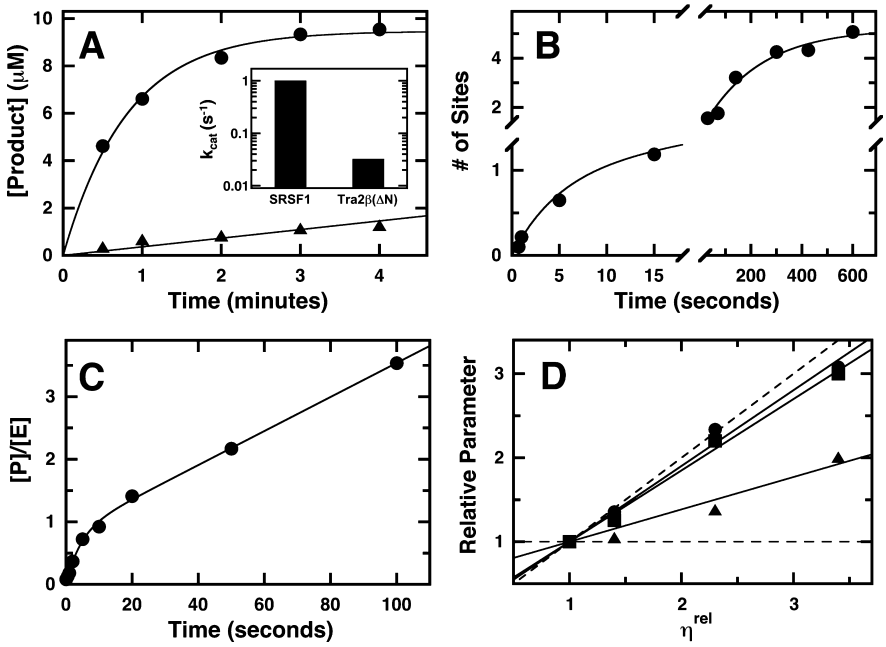

Figure 2. Measuring the phosphoryl transfer step to Tra2β(ΔN). (A) Steady-state progress curves. SRPK1 (200 nM) is mixed with 1 μM SRSF1 (●) and Tra2β(ΔN) (▲). For SRSF1, the data are fit to a single exponential function with an amplitude and rate constant of 9.5 ± 0.2 μM and 1.2 ± 0.10 min^{-1}. For Tra2β(ΔN), the data are fit to a linear function with a slope of 0.36 ± 0.04 μM/min. The inset shows a comparison of k_{cat} obtained from plots of initial velocity versus both substrates. (B) Single-turnover analyses. SRPK1 (2 μM) and Tra2β(ΔN) (0.1 μM) are mixed with ATP (100 μM) in the rapid quench-flow machine, and the data are fit to a double exponential function with amplitudes of 0.9 ± 0.2 and 4.3 ± 0.3 sites and rate constants of 0.21 ± 0.10 and 0.005 ± 0.001 s^{-1}. (C) Pre-steady-state kinetic analyses. SRPK1 (0.25 μM) and Tra2β(ΔN) (1 μM) are mixed with ATP (100 μM) in the rapid quench-flow machine, and the data are fit to eq 1 to obtain values of 0.82 ± 0.08, 0.23 ± 0.04 s^{-1}, and 0.027 ± 0.008 s^{-1} for α, k_b, and k_L, respectively. (D) Viscosity effects on k_{cat} (●), k_{cat}/K_{SR} (■), and k_{cat}/K_{ATP} (▲). Parameters are displayed as a ratio in the absence and presence of varying sucrose concentrations against the relative buffer viscosity (η^{rel}). The slopes for k_{cat} $[(k_{cat})^{\eta}]$, k_{cat}/K_{SR} $[(k_{cat}/K_{SR})^{\eta}]$, and k_{cat}/K_{ATP} $[(k_{cat}/K_{ATP})^{\eta}]$ are 0.90 ± 0.04, 0.85 ± 0.03, and 0.40 ± 0.04, respectively. In the absence of viscosogens, values of 0.032 ± 0.003 s^{-1}, 0.91 ± 0.20 μM^{-1} sec^{-1}, 2.9 ± 0.5 mM^{-1} sec^{-1} were obtained for k_{cat}, k_{cat}/K_{SR}, and k_{cat}/K_{ATP}, respectively. The dotted lines represent theoretical slope values of 0 and 1.

terminal half of the RS domain is a major site for other splicing kinases, we investigated the phosphorylation of Tra2β(ΔN) by CLK1, which phosphorylates both Arg−Ser and Ser−Pro dipeptides in SR proteins.[21] We found that CLK1 modifies about three additional serines in Tra2β(ΔN) compared to SRPK1, which likely corresponds to the three Ser−Pro dipeptides in the RS domain (Figure 1 A,G). CLK1 modifies only two-to-three serines in Tra2β(ΔN-4RA), suggesting that CLK1 is targeting the same Arg−Ser repeat as SRPK1. This is further supported by the observation that Tra2β(ΔN/RS2) is very weakly phosphorylated by CLK1 (Figure 1G). Overall, the data are consistent with a common region of phosphorylation corresponding to the $(RS)_4$ repeat and an isolated dipeptide in the C terminus of the Tra2β(ΔN) RS domain

Phosphoryl Transfer Step Does not Limit Tra2β(ΔN) Turnover. The phosphoryl transfer step in SRPK1 is very fast and does not limit turnover of the SR protein SRSF1.[17] Because this substrate contains a long Arg−Ser repeat that may facilitate correct alignment of the RS domain and rapid turnover (Figure 1A), we wondered whether the corresponding RS domain in Tra2β(ΔN), which lacks a lengthy repeat segment, would be efficiently modified in the active site of SRPK1. In steady-state progress curves with equal amounts of enzyme and saturating substrate, Tra2β(ΔN) is turned over by SRPK1 at a much slower rate than SRSF1 (Figure 2A). These differences correspond to decreases in k_{cat} from 1 s^{-1} for SRSF1 to 0.03 s^{-1} for Tra2β(ΔN) (Figure 1A, inset). To determine whether the reduced turnover number for Tra2β(ΔN) is the result of an impaired phosphoryl transfer step, we performed rapid quench-flow experiments to isolate the first phosphorylation event. In single-turnover experiments, we showed that Tra2β(ΔN) phosphorylation occurs in two discrete kinetic phases (Figure 2B). The initial phase (0.2 s^{-1}) includes the first phosphorylation event and is about 7-fold faster than k_{cat}, suggesting that the phosphoryl transfer step is fast relative to turnover. Interestingly, although k_{cat} mostly reflects early phosphorylation events, the initial velocity of the second phase in the single-turnover experiment (0.02 sites s^{-1}) is close to k_{cat}, suggesting that a common rate-limiting step may control multisite phosphorylation in Tra2β(ΔN). To confirm that the phosphoryl transfer step is fast, we performed pre-steady-state kinetic experiments and found that the kinetic profile is consistent with a rapid phosphoryl transfer step or burst phase preceding the linear steady-state phase (Figure 2C). The data were fit to eq 1 to obtain a burst rate constant (k_b) of 0.2 s^{-1}, a value identical to that observed in the first phase of the single-turnover experiment. Also, the linear rate constant (k_L = 0.027 s^{-1}) is close in value to k_{cat}, indicating that the experiment also captures the steady-state phase detected at low enzyme concentration. Overall, these data suggest that the phosphoryl transfer step is fast for SRPK1 and does not limit net turnover of Tra2β(ΔN).

Viscosity-Dependent Step Regulates Tra2β(ΔN) Phosphorylation. Because the phosphoryl transfer step to Tra2β(ΔN) is fast, we wished to determine whether the release of a reaction product could limit k_{cat}. To investigate this

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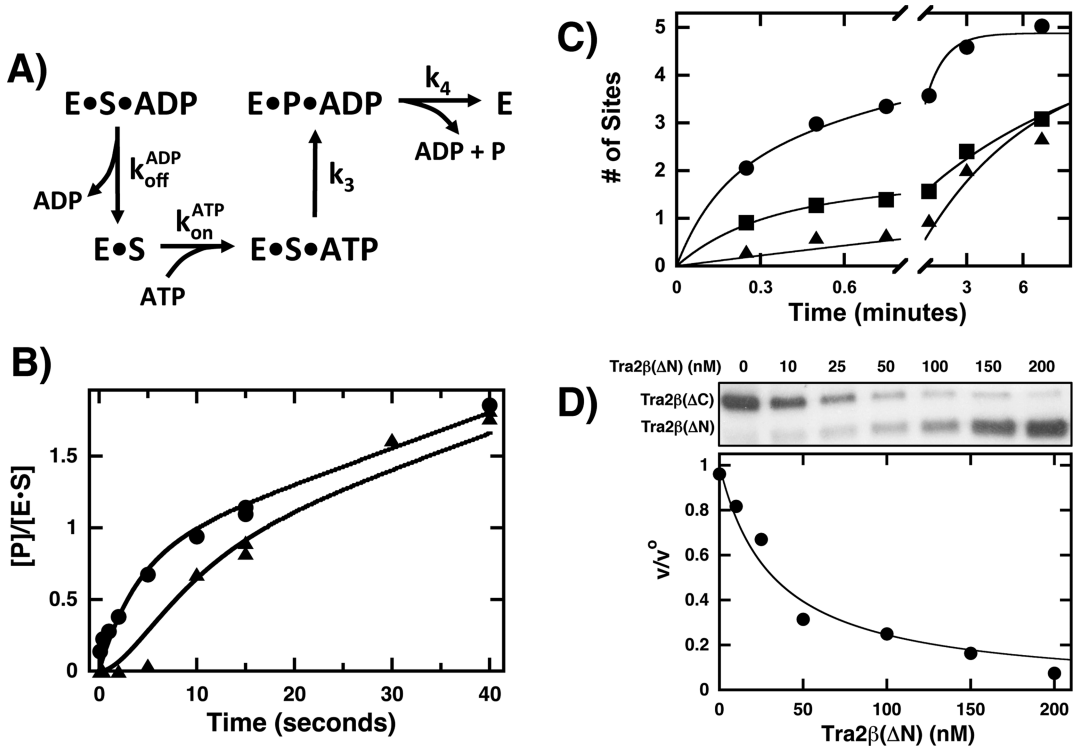

Figure 3. Analysis of product release for Tra2β(ΔN) turnover. (A) Mechanism used to analyze the kinetic data experiment. S = Tra2β(ΔN), P = phospho-Tra2β(ΔN), and E = SRPK1. (B) $C_{AT}T_{RAP}$ experiment. SRPK1 is preincubated with Tra2β(ΔN) in the absence (●) and presence (▲) of 120 μM ADP in one syringe (60 μM in reaction) and then mixed with ATP (600 μM in reaction) to start the reaction. The data are simulated using DynaFit[33] and the mechanism in panel A to obtain values of 0.34, 0.23, and 0.024 s^{-1} for k_{off}, k_3, and k_4, respectively. A value of 7 mM^{-1} sec^{-1} for the ATP k_{on} was used for both simulations. (C) Start-trap experiment. A complex of SRPK1 (1 μM) and Tra2β(ΔN) (0.2 μM) is mixed with ATP (60 μM) in the absence (●) and presence (■) of kdSRPK1 (60 μM). In a control experiment (trap-start), the complex is pre-equilibrated with kdSRPK1 before reaction initiation with ATP (▲). The reaction in the absence of kdSRPK1 is fit to a double exponential function with amplitudes of 1.40 ± 0.14 and 3.40 ± 0.13 sites and rate constants of 9 ± 5 and 1 ± 0.15 min^{-1}, respectively. The start-trap data are fit to a double exponential function with amplitudes of 1.3 ± 0.15 and 3.7 ± 0.20 sites and rate constants of 3.7 ± 1.2 and 0.10 ± 0.01 min^{-1}, respectively. The trap-start data are fit to a single exponential function with an amplitude of 4.5 ± 0.5 sites and a rate constant of 0.15 ± 0.02 min^{-1}. (D) Competition experiment. SRPK1 (5 nM), Tra2β(ΔC) (500 nM), ATP (60 μM), and varying amounts of Tra2β(ΔN) (0–200 nM) are allowed to react for 2.5 min. The relative velocity for Tra2β(ΔC) obtained from the autoradiogram is plotted as a function of Tra2β(ΔN) and fitted to eq 6 to obtain a K_I of 11 ± 2 nM for Tra2β(ΔN) using a K_m of 270 nM for Tra2β(ΔC) measured in separate experiments.

question, we initially performed viscosometric experiments. If the release of one or both of the products [ADP or phospho-Tra2(ΔN)] controls substrate phosphorylation, then k_{cat} should decline with increasing solvent viscosity according to the Stokes–Einstein relationship.[28,29] Using a fixed amount of Tra2β(ΔN), we measured the initial velocity as a function of ATP in the presence of varying amounts of sucrose. The relative k_{cat} (the ratio of k_{cat} in the absence and presence of viscosogen) increases as a function of the relative viscosity with a slope value [$(k_{cat})^{\eta}$] of 0.9 (Figure 2C). Because this value is close to the theoretical upper limit of 1, the release of one or both of the reaction products is likely to limit k_{cat}. In previous studies, we showed that the net release of products (k_4) and the phosphoryl transfer step (k_3) in a protein kinase reaction can be estimated from k_{cat} and $(k_{cat})^{\eta}$ according to eqs 2 and 3.[28,29] Using this approach, values of 0.32 and 0.036 s^{-1} can be derived for k_3 and k_4. The former value is similar to the phosphoryl transfer rate constant from the rapid quench-flow experiments (Figure 2A,B). These data suggest that Tra2β(ΔN) turnover is limited by a viscosity-dependent step that most likely corresponds with product release.

Investigating ADP Release Rate using $C_{AT}T_{RAP}$ Experiments. Because ADP release limits multisite phosphorylation of SRSF1 by SRPK1,[16,17] we asked whether a similar product dissociation step could control Tra2β(ΔN) turnover. To accomplish this, we employed a catalytic-trapping ($C_{AT}T_{RAP}$) experiment[30] in which the SRPK1–Tra2β(ΔN) complex is first preincubated with ADP before reaction initiation with excess ATP in the rapid quench-flow instrument (Figure 3A). Using this methodology, we found that ADP preincubation resulted in a small 'lag' relative to the control lacking ADP before resumption of product formation (Figure 3B). Two criteria are used to verify that this sigmoidal behavior is not the result of (1) inadequate initial complex formation with ADP or (2) inefficient trapping of the ADP-free complex with ATP.[28] First, because the total ADP concentration is more than 10-fold above its K_I,[17] most of the initial enzyme–substrate complex should be saturated with ADP prior to ATP addition. Also, if some of the complex lacked bound ADP, then a small burst phase would be detected rather than a lag phase, but this is not the case. Second, because the linear rates of the reaction with and without ADP are similar (t > 15 s), sufficient ATP is added to compete with ADP and to achieve maximum turnover. These observations establish that SRPK1 initially forms a complex with ADP and that enough ATP is present to trap this complex in the rapid quench experiment.

To analyze the $C_{AT}T_{RAP}$ experiments, the data were simulated using the mechanism in Figure 3A. The association

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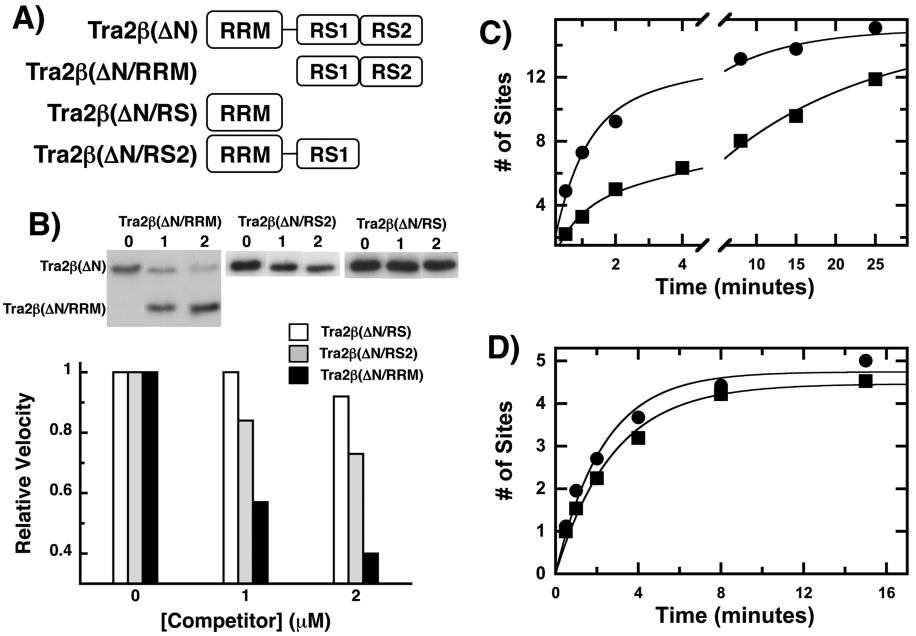

Figure 4. Polypeptide segments important for SRPK1-Tra2β(ΔN) interactions. (A) Deletion constructs. (B) Binding of Tra2β(ΔN) deletions. Relative initial velocities for Tra2β(ΔN) (1 μM) phosphorylation are obtained from the autoradiogram and are displayed as a function of 1 or 2 μM of the following competitors: Tra2β(ΔN/RS), Tra2β(ΔN/RRM), and Tra2β(ΔN/RS2). (C) Progress curves for the phosphorylation of SRSF1 by SRPK1 (●) and SRPK1(6M) (■). The data are fit to a double exponential function to obtain amplitudes and rate constants of 10 ± 1 and 5 ± 1 sites and 1.2 ± 0.1 and 0.11 ± 0.02 min^{-1} for SRPK1 and amplitudes and rate constants of 4 ± 0.5 and 11 ± 0.5 sites and 1.3 ± 0.2 and 0.050 ± 0.022 min^{-1} for SRPK1(6M). (D) Progress curves for the phosphorylation of Tra2β(ΔN) by SRPK1 (●) and SRPK1(6M) (■). The data are fit to a single exponential function to obtain an amplitude and rate constant of 4.7 ± 0.2 sites and 0.44 ± 0.06 min^{-1} for SRPK1 and an amplitude and rate constant of 4.5 ± 0.2 sites and 0.36 ± 0.04 min^{-1} for SRPK1(6M).

rate constant for ATP (k_{on}^{ATP}) was determined from the ratio of k_{cat}/K_m for ATP (k_{cat}/K_{ATP}; 2.9 mM^{-1} sec^{-1}) and the viscosity sensitivity on this parameter $(k_{cat}/K_{ATP})^{\eta}$ (Figure 2C) using eq 4 and was set constant for the simulations (7 mM^{-1} sec^{-1}). After fixing k_{on}^{ATP}, we obtained an optimal simulation for the kinetic data with values of 0.23 and 0.028 s^{-1} for k_3 and k_4 in the absence of ADP. We then simulated the data with ADP pre-equilibration and obtained optimal fitting using a value of 0.34 s^{-1} for the ADP dissociation rate constant (k_{off}^{ADP}). This rate constant is close to a prior value (1 s^{-1}) using SRSF1 as a substrate in the $C_{AT}T_{RAP}$ experiment.[17] Because k_{off}^{ADP} is about 7-fold higher than k_{cat}, ADP release does not limit net Tra2β(ΔN) phosphorylation. To provide further evidence that nucleotides exchange rapidly in the active site relative to k_{cat}, we calculated the dissociation rate constant for ATP (k_{off}^{ATP}) using k_{cat}, $(k_{cat})^{\eta}$, $(k_{cat}/K_{ATP})^{\eta}$, and eq 5. With this analysis, we can estimate a value of 0.48 s^{-1} for k_{off}^{ATP} from the enzyme–substrate complex, a value close to k_{off}^{ADP} obtained from the $C_{AT}T_{RAP}$ experiments. In summary, these experiments show that the release of ADP from SRPK1 is faster than k_{cat} and does not control the rate of Tra2β(ΔN) turnover.

Monitoring Tra2β(ΔN) Release using Start-Trap Experiments. The rapid quench-flow experiments show that the phosphoryl transfer and ADP release steps are fast, suggesting that phospho-Tra2β(ΔN) dissociation controls the overall turnover. To address this, we performed a start-trap experiment to evaluate whether protein dissociation occurs during normal catalytic cycling. In this experiment, the enzyme–substrate complex is mixed with ATP in the absence and presence of a kinase-inactive form of SRPK1 (kdSRPK1).[14] In prior studies, we showed that a K → M mutation in the active site of SRPK1 generates an inactive kinase that binds tightly to SRSF1 and inhibits its phosphorylation.[19] In the start-trap experiment, if any phospho-forms of Tra2β(ΔN) are released from the active site during the reaction, then kdSRPK1 will trap them and inhibit further phosphorylation. However, if Tra2β(ΔN) is phosphorylated without dissociation from SRPK1 in a processive manner, then kdSRPK1 will not affect the reaction. To evaluate whether the inactive kinase is an effective trap for Tra2β(ΔN), we preincubated kdSRPK1 with the SRPK1–Tra2β(ΔN) complex before the addition of ATP. In this trap-start experiment, kdSRPK1 strongly inhibited Tra2β(ΔN) phosphorylation compared to the control reaction lacking kdSRPK1 (Figure 3C). On the basis of the initial portion of the progress curve, the reaction velocity is at least 10-fold slower in the presence of kdSRPK1. When kdSRPK1 is added simultaneously with ATP in the start-trap experiment, it also strongly inhibited the progress curve compared to the control reaction lacking kdSRPK1 (Figure 3C). This result is consistent with a mechanism in which Tra2β(ΔN) is released after each round of phosphorylation in a distributive manner. These findings suggest that phospho-Tra2β(ΔN) dissociation could play a role in controlling net SR protein turnover.

In the start-trap experiment, we observed a small, rapid generation of phospho-Tra2β(ΔN) in the earliest time points equivalent to roughly one site relative to the control reaction (Figure 3C). This may be the result of slow exchange of Tra2β(ΔN) from the enzyme–substrate complex relative to forward catalysis. To attain additional information on how fast Tra2β(ΔN) exchanges with SRPK1, we measured the dissociation rate constant for the substrate using the viscosity-dependent data in Figure 2D. Substituting $(k_{cat}/K_{SR})^{\eta}$ for $(k_{cat}/K_{ATP})^{\eta}$ in eq 5, we estimated a k_{off} for Tra2β(ΔN) of 0.06 s^{-1}, a value close to k_{cat}. This exchange rate for the protein substrate

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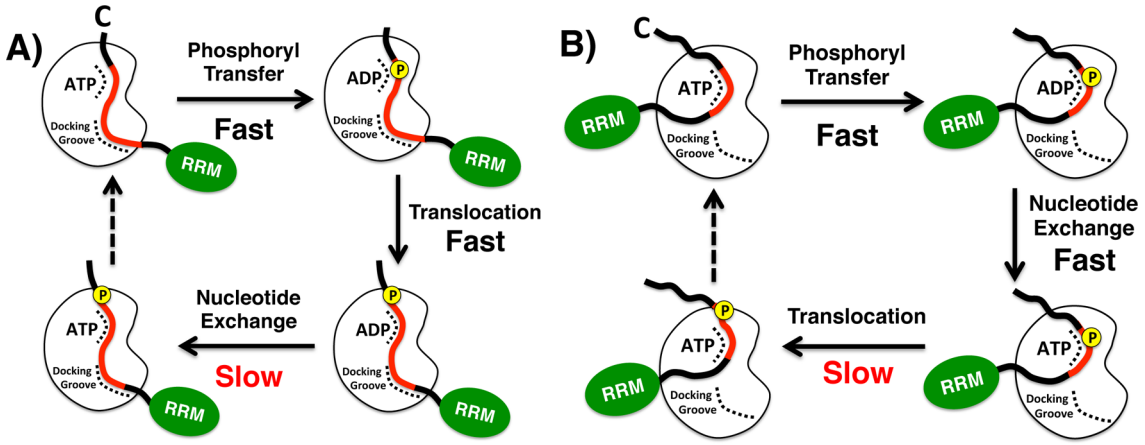

Figure 5. Mechanisms for RS domain phosphorylation. (A) Long Arg−Ser repeats. The docking groove orients the RS domain for efficient phosphoryl transfer and translocation of N-terminal dipeptides into the active site. The rate-limiting step is ADP release (nucleotide exchange). (B) Short Arg−Ser repeats. The docking groove is bypassed and only local residues in the active site control RS domain phosphorylation. Both the phosphoryl transfer and nucleotide exchange steps are fast, whereas the translocation step is slow. The red portions of the RS domains designate Arg−Ser-rich regions.

is slower than k_3 and could account for the rapid generation of phospho-product in the initial phase of the start-trap experiment. To verify further that Tra2β(ΔN) dissociates slowly from SRPK1, we measured its affinity in a competition experiment.[14] In this method, we measured the phosphorylation velocity of a fixed amount of a truncated form of Tra2β that lacks the C-terminal RS domain but maintains the N-terminal RS domain (Tra2β(ΔC)) as a function of increasing Tra2β(ΔN). The phosphorylation of the alternate substrate Tra2β(ΔC) declines as that for Tra2β(ΔN) increases (Figure 3D). By fitting the data to a standard equation for competitive inhibition (eq 6), we obtained a K_I for Tra2β(ΔN) of 11 nM, a value close to the substrate K_m of 35 nM (Figure 3D). Using the association rate constant for Tra2β(ΔN) determined from the viscosity-dependent data (1.1 μM^{-1} sec^{-1}), we can then use the K_I to estimate a value of 0.012 s^{-1} for k_{off}, a value close to k_{cat}. Overall, the data garnered from rapid quench-flow, start-trap, competition, and viscosity experiments support a model where the phosphoryl transfer and ADP release steps are fast and protein product dissociation is a necessary step after each round of phosphorylation. This protein release step is slow and is likely to limit the net rate of Tra2β(ΔN) catalysis.

RS Domain Drives High-Affinity Binding of Tra2β(ΔN). To determine what structural factors govern high-affinity binding of Tra2β(ΔN), we studied the binding of several truncated forms of this substrate (Figure 4A) using a competition assay. In this experiment, we monitored the phosphorylation of a fixed amount of Tra2β(ΔN) with increasing Tra2β(ΔN/RRM), an alternate substrate, and two nonphosphorylatable forms of Tra2β(ΔN) (Tra2β(ΔN/RS2) and Tra2β(ΔN/RS)) (Figure 4B). In general, we discovered that sequences containing the RS domain are important for inhibiting phosphorylation of the control substrate Tra2β(ΔN). By fitting the velocity data to eq 6, we found that the full RS domain (Tra2β(ΔN/RRM)) binds well to SRPK1 with a K_I of 45 nM, a value similar to the substrate K_m and only 4-fold higher than the K_I for Tra2β(ΔN). In comparison, the construct lacking the RS domain (Tra2β(ΔN/RS)) did not inhibit Tra2β(ΔN) phosphorylation (Figure 4A,B), implying that the free RRM does not interact well with SRPK1. These findings suggest that the RS domain mostly drives high-affinity binding of Tra2β(ΔN). Because only a portion of the RS domain is phosphorylated by SRPK1, we wished to determine whether either half of the RS domain participates in high-affinity interactions by measuring the binding affinity of Tra2β(ΔN/RS2) in which the C-terminal portion of the RS domain is deleted. We found that Tra2β(ΔN/RS2) could inhibit Tra2β(ΔN) with a K_I of 180 nM, a value 5-fold higher than the substrate K_m and 16-fold higher than the K_I for Tra2β(ΔN). In summary, these findings indicate that RS domain drives high-affinity binding of Tra2β(ΔN) to SRPK1. Although the N terminus provides some stability, the C terminus of the RS domain is the main driver of high-affinity binding of Tra2β(ΔN).

Tra2β(ΔN) Bypasses the Docking Groove in SRPK1 for RS Domain Phosphorylation. Rapid phosphorylation of SRSF1 is dependent on an electronegative docking groove in the large lobe of the kinase domain of SRPK1 that binds N-terminal Arg−Ser repeats prior to translocation into the active site[15] (Figure 5A). Mutations in this docking groove result in a less efficient kinase that randomly phosphorylates the RS domain of SRSF1.[14] To determine whether this groove plays a role in phosphorylating Tra2β(ΔN), we performed single-turnover experiments using a mutant form of SRPK1 in which six electronegative residues in the groove are replaced with alanine (SRPK1(6M)).[14] As previously observed, SRSF1 phosphorylation is reduced for SRPK1(6M) owing to a large decrease in the amplitude for the initial phase compared to the wild-type kinase (Figure 4C). These findings show that fast multisite phosphorylation of SRSF1 is facilitated by a functioning docking groove in SRPK1. In comparison, Tra2β(ΔN) was phosphorylated at similar rates by SRPK1-(6M) and SRPK1 (Figure 4D). Also, mutation of the docking groove had no impact on the net phosphoryl content of Tra2β(ΔN) in the time frame of the assay. Both SRPK1 and SRPK1(6M) added about five phosphates onto Tra2β(ΔN). Overall, these findings suggest that, unlike SRSF1, Tra2β(ΔN) does not require a functioning docking groove in SRPK1 for efficient RS domain phosphorylation.

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DISCUSSION

SRPK1 phosphorylates a strand of 11 consecutive serines in the RS domain of SRSF1 using a novel sequential mechanism where the kinase moves in a strict N-terminal direction.[13,31] Phosphorylation initiation is enforced by a conserved, electronegative docking groove in the large lobe of the kinase domain that binds N-terminal dipeptides and positions the C-terminal dipeptides in the active site for the first round of catalysis (Figure 5A). Arg−Ser dipeptides in the RS domain then move from the docking groove to the active site in a stepwise manner until the entire strand is phosphorylated. Prior studies indicate that about five-to-eight serines are phosphorylated without dissociation of the SR protein.[13] This processive phase is transitory because the stability of the SRPK1−SRSF1 complex declines with the increasing phosphoryl content of the RS domain, thus facilitating protein dissociation in the late stages of the reaction.[17] Mutagenesis studies have shown that both directionality and processive phosphorylation is dependent on a functioning docking groove.[14] Despite its apparent complexity, the phosphorylation of the RS domain in SRSF1 is highly efficient. The individual catalytic and dipeptide translocation steps are very fast, implying that movement of the RS domain does not present a significant barrier. Rather, the slow release of ADP (nucleotide exchange) controls multisite phosphorylation of this SR protein.[17] Although SRSF1 contains a very long Arg−Ser strand that is capable of spanning both the docking groove and active site, many SR proteins contain much shorter repeat regions, raising the question of how these substrates are processed by SRPK1.

Adapting to Other RS Domain Configurations. Although many SR proteins present a mix of long and short Arg−Ser repeats (eg., SRSF1 and SRSF2), some lack any repeats above five or six dipeptides (Figure S1). For example, SRSF4, 6, 7, and 10 contain Arg−Ser repeats of one-to-four dipeptides in length, raising the question of how such SR proteins are activated by SRPK1 given the length constraints imposed by the docking groove and the active site. Using Tra2β(ΔN) as a substrate, we investigated the consequences of an RS domain with more truncated Arg−Ser segments in light of the phosphorylation model established for SRSF1 (Figure 5A). Through detailed kinetic analyses, we found that the mechanism of phosphorylation shifts substantially with the nature of the RS domain. Although the longer repeat in SRSF1 is phosphorylated using several processive steps that are limited by ADP release, Tra2β(ΔN) is phosphorylated in a distributive manner where protein release appears to control each round of phosphorylation (Figure 5B). Of critical importance is the observation that conserved elements of the kinase structure are dispensable depending on the RS domain. Although the docking groove in SRPK1 is essential for rapid, directional phosphorylation of SRSF1,[14] it appears to be unnecessary for Tra2β(ΔN) phosphorylation (Figure 4D). Such a finding suggests that the N-terminal portion of the RS domain may not reside in the docking groove or that its presence has no impact during multiple catalytic cycles (Figure 5B). These results strongly indicate that the docking groove may be important for the phosphorylation of lengthy Arg−Ser repeats but may be circumvented for shorter Arg−Ser repeats.

Our new kinetic data indicate that changes in the RS domain landscape have profound effects on the phosphorylation mechanism. Although RS domain translocation is fast and ADP release slow for SRSF1 phosphorylation, the rate-limiting step for Tra2β(ΔN) phosphorylation is protein release. A critical question is how does SRPK1 process the two RS domains using such different kinetic parameters yet bind the SR proteins with equivalent affinities? The answer to this question lies in how SRPK1 utilizes its docking groove for these two substrates. Previous studies showed that although it is essential for rapid processive phosphorylation of SRSF1, the docking groove plays no significant role in controlling overall binding affinity.[14] Thus, the groove largely serves a kinetic function and, as such, offers a low-energy barrier for transferring Arg−Ser repeats to the active site compared to the alternative option of successive SRSF1 dissociation steps. However, for Tra2β(ΔN), which does not use the docking groove, the contacts for high affinity do not provide a low-energy pathway for feeding dipeptides into the active site. From these findings, we speculate that for RS domains with short Arg−Ser repeats that cannot occupy the docking groove the protein substrate may be required to dissociate and rebind after each round of phosphorylation, a process that is less efficient and leads to low turnover. Furthermore, given the wide diversity of short versus long repeats (Figure S1), it is worth speculating that processive phosphorylation could be isolated to SR proteins with long Arg−Ser dipeptide repeats (e.g., SRSF1 and SFSF2), whereas distributive phosphorylation could be the dominant phosphorylation mode in SR proteins with shorter repeats (e.g., SRSF4, 6, 7, and 10).

Attaining High-Affinity Binding through Flexible Binding Contacts. Deletion analyses indicate that the high-affinity contacts for Tra2β(ΔN) are mostly localized to the C-terminal half of the RS domain. This contrasts with SRSF1 where high-affinity binding lies in the N terminus of the RS domain.[14] An explanation for such a contrasting mode of recognition for these two substrates may be gleaned from a comparison of the RS domains. Although both the N- and C-terminal halves of the SRSF1 and Tra2β(ΔN) RS domains are equally arginine-rich, the N-terminal half of the Tra2β(ΔN) RS domain is uniquely rich in negatively charged residues with seven aspartates (Figure 1A). These charges may counteract the arginines in this region, depriving SRPK1 of enough stable electrostatic contacts for binding the N terminus of the RS domain. Indeed, these negative charges may explain both the reliance on C-terminal residues for high-affinity binding and the inability to phosphorylate the N-terminal serines in the RS domain of Tra2β(ΔN). For SRSF1, which lacks negatively charged residues in its RS domain, all serines flanking arginines are phosphorylated by SRPK1.[14] However, the RS domain in SRSF1 is not typical with regard to charge distribution. It is worth noting that some SR proteins have negatively charged residues (e.g., SRSF4 has six aspartates and 21 glutamates) that could limit phosphorylation of one or more discrete Arg−Ser repeats (Figure S1). The potential to turn off several sites in Tra2β(ΔN) suggests that RS domain phosphorylation could be more complicated than originally thought and that a combination of Arg−Ser position in the context of other controlling residues may generate alternative phosphorylation patterns within the larger SR protein family. Finally, although C-terminal residues in the Tra2β(ΔN) RS domain are the main drivers of high affinity, the N-terminal residues provide some stability. Although we do not think that these residues occupy the docking groove, they could bind to other surfaces on SRPK1. This raises the exciting possibility that different RS domains not parsed through the conserved docking groove may

interact at other locations outside the active site, thereby uniquely contributing to multisite phosphorylation.

CONCLUSIONS

Much of what we know about RS domain phosphorylation stems from studies on what is considered the prototype for the family, SRSF1. The protein kinase SRPK1 uses a conserved docking groove to efficiently phosphorylate the RS domain of this SR protein using a semiprocessive, directional mechanism (Figure 5A). Because most SR proteins contain short Arg−Ser repeats, we explored how a change in the RS domain landscape would affect this mechanism. We found that shifting to a shorter repeat had profound affects on kinetic processing. Rather than occupying the docking groove as in the case of SRSF1, the RS domain of Tra2β(ΔN) appears to bypass this conserved structural element (Figure 5B). The price of phosphorylating this shorter Arg−Ser repeat is a shift in rate-limiting step from slow nucleotide to protein release and a concomitant change from semiprocessive to distributive phosphorylation. Any binding in the docking groove with longer repeats greatly increases phosphorylation efficiency through the feeding mechanism without significantly improving binding affinity. Thus, the role of the docking groove is one of organization rather than stability. These findings raise the possibility that the docking groove might untangle unproductive structure within lengthy Arg−Ser repeats so that they can be efficiently phosphorylated. Prior computational studies suggest that a peptide of eight Arg−Ser repeats adopts a helical conformation that may be difficult to phosphorylate without the organizing function of a docking groove.[32] In contrast, shorter Arg−Ser repeats may be more unstructured and therefore may not require the docking groove. Overall, these findings point to a highly flexible kinase that can readily adopt to a changing RS domain architecture.

ASSOCIATED CONTENT

Supporting Information

RS domain sequences of several SR proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

$C_{AT}T_{RAP}$, catalytic trapping; CLK1, Cdc2-like kinase 1; RRM, RNA recognition motif; RS domain, domain rich in arginine−serine repeats; SR protein, splicing factor containing arginine−serine repeats; SRPK1, SR-specific protein kinase 1; SRSF1, SR protein splicing factor 1 (aka ASF/SF2); Tra2β, transformer 2β protein

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