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Intra-domain Cross-talk Regulates Serine-arginine Protein Kinase 1-dependent Phosphorylation and Splicing Function of Transformer 2β1*

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Background: Serine-arginine-rich (SR)-like proteins regulate the alternative splicing of human genes.
Results: The serine-arginine protein kinase 1 (SRPK1) phosphorylates transformer 2β1 (Tra2β1) at numerous sites regulating RNA binding, splicing of the survival motor neuron 2 gene, and catalytic function of the kinase domain.
Conclusion: The two RS domains interact and regulate Tra2β1 activity in a phosphorylation-dependent manner.
Significance: SRPK1 is a regulator of Tra2β1.

Transformer 2β1 (Tra2β1) is a splicing effector protein composed of a core RNA recognition motif flanked by two arginine-serine-rich (RS) domains, RS1 and RS2. Although Tra2β1-dependent splicing is regulated by phosphorylation, very little is known about how protein kinases phosphorylate these two RS domains. We now show that the serine-arginine protein kinase-1 (SRPK1) is a regulator of Tra2β1 and promotes exon inclusion in the survival motor neuron gene 2 (*SMN2*). To understand how SRPK1 phosphorylates this splicing factor, we performed mass spectrometric and kinetic experiments. We found that SRPK1 specifically phosphorylates 21 serines in RS1, a process facilitated by a docking groove in the kinase domain. Although SRPK1 readily phosphorylates RS2 in a splice variant lacking the N-terminal RS domain (Tra2β3), RS1 blocks phosphorylation of these serines in the full-length Tra2β1. Thus, RS2 serves two new functions. First, RS2 positively regulates binding of the central RNA recognition motif to an exonic splicing enhancer sequence, a phenomenon reversed by SRPK1 phosphorylation on RS1. Second, RS2 enhances ligand exchange in the SRPK1 active site allowing highly efficient Tra2β1 phosphorylation. These studies demonstrate that SRPK1 is a regulator of Tra2β1 splicing function and that the individual RS domains engage in considerable cross-talk, assuming novel functions with regard to RNA binding, splicing, and SRPK1 catalysis.

The splicing of precursor mRNA transcripts is dependent on an essential group of splicing factors known as SR[3] proteins.

These factors bind near exon-intron boundaries facilitating the association of U1 small nuclear ribonucleoprotein and U2AF, two early components that initiate assembly of the macromolecular spliceosome (1, 2). SR proteins also participate in other steps along the assembly pathway, including the final step that generates the active spliceosome (3, 4). SR proteins contain one or two RNA recognition motifs (RRMs) that bind exonic splicing enhancer (ESEs) sequences near the splice junctions and a C-terminal Arg-Ser-rich domain (RS domain) that regulates the former. Although RRMs are compact, folded domains, RS domains are considered intrinsically disordered (5, 6). The facilitation of U1 small nuclear ribonucleoprotein and U2AF attachment to transcripts is dependent on RS domain phosphorylation making SR proteins and their post-translational modifications necessary for constitutive mRNA splicing (1, 2). Recent studies suggest that phosphorylation is likely to induce a conformational change in one SR protein (SRSF1 (SR protein splicing factor 1 (also known as ASF/SF2)) that exposes its RRM-promoting interactions with an RRM from the 70K subunit of U1 small nuclear ribonucleoprotein (7). Although this provides a simple mechanism for exon definition, the link between splice-site selection and phosphorylation appears to be much more complex. For example, it has been shown that varying levels of SR protein phosphorylation correlate strongly with changes in alternative splicing, a process whereby 5′ and 3′ splice-site regulation generates multiple mRNA strand isoforms (8 –11). Although alternative splicing is a remarkable mechanism for proteome diversity and organismal complexity, little is know about the rules of RS domain phosphorylation and how graded levels of phosphorylation regulate this process.

Most SR proteins are thought to be poly-phosphorylated based on numerous Arg-Ser dipeptides within the RS domain and a strong reactivity with a phosphorylation-sensitive monoclonal antibody, mAb104 (12). Although several protein kinases phosphorylate SR proteins, two prominent kinase families

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[3] The abbreviations used are: SR, splicing factor containing arginine-serine repeat; ESE, exonic splicing enhancer; kdSRPK1, kinase-dead SRPK1 with K109M, MALDI-TOF, matrix assisted laser desorption/ionization-time of flight; RRM, RNA recognition motif; RS domain, domain rich in arginine-

serine repeats; RS1, N-terminal RS domain of Tra2β1; RS2, C-terminal RS domain of Tra2β1; SMN, survivor motoneuron gene; SRPK1(6M), SRPK1 with six mutations in docking groove.

(SRPKs and CLKs) have been linked to SR protein-dependent splicing (8, 9, 13). In recent years, peptide mapping and kinetic methods have been employed to better define the phosphorylation state and mechanism for SRSF1. The RS domain in this splicing factor is the smallest within the SR protein family (50 residues) and contains both long and short Arg-Ser dipeptide repeats that are phosphorylated differentially. SRPK1 (serine-arginine protein kinase 1) efficiently targets a long repeat region in the N-terminal half of the RS domain phosphorylating it in a strict C- to N-terminal direction (14). This unique mechanism is semi-processive, and is driven by an electronegative docking groove in the kinase domain that sequentially feeds dipeptides into the active site (14–16). SRPK1 can rapidly phosphorylate about 9–12 serines on this Arg-Ser dipeptide stretch, a modification that directs SRSF1 attachment to a specific transportin (TRN-SR) and subsequent entry into the nucleus (17, 18). Detailed structure-function studies indicate that SRPK1 mostly targets longer Arg-Ser repeats in the RS domain of SRSF1 (15). Although these studies have opened a window into RS domain phosphorylation, SRSF1 is one of 12 members of the SR protein family (SRSF1–12) that differ considerably in the position, number, and length of their Arg-Ser repeat regions (19). How SRPKs target these diverse RS domains and affect splicing function is still not understood.

Several splicing factors depart from the traditional SR proteins and fall into the category of SR-like proteins. Unlike classic SR proteins, SR-like proteins do not complement SR protein-deficient HeLa cell S100 extracts indicating that they are not essential for splicing (20). However, they control splice-site usage and antagonize the actions of traditional SR proteins (21). One member of the SR-like family is transformer 2 (Tra2), a protein first identified as a sex-determining factor in *Drosophila* (22). Although human Tra2 contains an RRM and a C-terminal RS domain, it also contains a long, N-terminal RS domain distinguishing it from the traditional SR proteins. One isoform, Tra2β1 (β1 isoform of Tra2 protein), has been shown to regulate gene splicing by binding to precursor mRNA and working cooperatively with other splicing factors, including SR proteins (23, 24). Tra2β1 has been implicated in neurodegenerative diseases by controlling specific exon usage in the *tau* gene. Several tauopathies, including frontotemporal dementia and Alzheimer disease correlate with a mis-balance in the 3R/4R isoforms of the Tau protein (13, 25). The up-regulation of Tra2β1 may have some therapeutic value in spinal muscle atrophy which results from a loss of the survival motor neuron-1 gene (*SMN1*). Patients have a second copy of the gene (*SMN2*) that does not rescue the disease phenotype because one exon (exon 7) is alternatively spliced producing a truncated, unstable protein (26). This alternatively spliced form of *SMN2* is the result of a single C-to-T change in the ESE of exon 7 (27). Interestingly, Tra2β1 expression promotes inclusion of exon 7 resulting in a full-length SMN protein (28).

Although Tra2β1 is important for splicing, the role of phosphorylation is still not well understood. Prior studies have shown that Tra2β1 hyper-phosphorylation, identified by a gel shift, inhibits RNA binding suggesting a role for kinase-mediated changes in spliceosome assembly and splice-site selection (29). Although SR protein phosphorylation facilitates nuclear

entry of SR proteins, hyper-phosphorylated forms of Tra2β1 are predominantly localized to the cytoplasm in the brain rather than the nucleus (30, 31). Furthermore, nuclear import of Tra2β1 by TRN-SR1 can occur in a phosphorylation-independent manner (32). Structure-function studies indicate that the N-terminal RS domain is necessary for Tra2β1 localization in nuclear speckles and that phosphorylation promotes cytoplasmic accumulation (31). These findings illustrate that the cytoplasmic-nuclear distribution of Tra2β1 is regulated differently than that for most SR proteins. However, despite its functional significance, little is know about the mechanism of Tra2β1 phosphorylation. In this study, we show that SRPK1 enhances Tra2β1-dependent splicing of the *SMN2* gene. Based on mass spectrometric and kinetic methods, we found that SRPK1 rapidly phosphorylates 21 serines in Tra2β1 using a docking groove in the kinase domain. Interestingly, although SRPK1 can phosphorylate five serines in the C-terminal RS domain (RS2) in the splice variant lacking RS1 (Tra2β3), the presence of RS1 in the full-length form inhibits RS2 phosphorylation suggesting RS domain cross-talk. Nonetheless, RS2 is not a silent domain. Based on viscosometric and rapid quench experiments, RS2 coordinately up-regulates phosphorylation rates in RS1 of Tra2β1 by increasing the association rate of the substrate and dissociation rate for ADP in SRPK1. The two RS domains serve coordinate roles in regulating SRPK1-dependent splicing and RNA binding. Although the unphosphorylated RS2 enhances binding to an ESE, the phosphorylated RS1 blocks this interaction. These studies provide the first detailed analysis of the phosphorylation of the SR-like protein Tra2β1. The results show that the two RS domains in this atypical splicing factor communicate and mutually regulate function.

Experimental Procedures

Materials—ATP, Mops, Tris, MgCl$_2$, NaCl, EDTA, glycerol, sucrose, acetic acid, Phenix imaging film, BSA, Whatman P81 grade filter paper, and liquid scintillant were obtained from Fisher. [γ-^{32}P]ATP was obtained from PerkinElmer Life Sciences. RNA (AAGAAC) was purchased from Integrated DNA Technologies; Hybond ECL nitrocellulose blotting membrane was purchased from Amersham Biosciences; KinaseMax^{TM} kit was purchased form Ambion; and Zip-tip C4 tips were purchased from Millipore. Phos-tag reagent was a generous gift from Dr. K.-L. Guan, University of California at San Diego.

Protein Expression and Purification—SRPK1, kdSRPK1, and SRPK1(6M) were expressed from a pET19b vector containing a His$_{10}$ tag at the N terminus (33). Tra2β1, Tra2β1(ΔRS1), and Tra2β1(ΔRS2) were expressed from a pET28a vector containing a C-terminal His$_6$ tag. All mutations were generated using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). All deletion constructs were generated using the Thermo Scientific Phusion site-directed mutagenesis kit (Integrated DNA Technologies, San Diego). The plasmids for wild-type and mutant forms of Tra2β1 and SRPK1 were transformed into the BL21 (DE3) *Escherichia coli* strain and grown at 37 °C in LB broth supplemented with 50 μg/ml kanamycin and 100 μg/ml ampicillin. Protein expression was induced with 1 μg/ml isopropyl 1-thio-β-D-galactopyranoside at room temperature for 4 h for all Tra2β1 proteins and with 2.5 μg/ml isopropyl 1-thio-β-D-

galactopyranoside for 12 h for SRPK1 proteins. SRPK1, SRPK1(6M), and all Tra2β1 proteins were purified by nickel resin affinity chromatography using previously published procedures (34).

SMN2 Minigene Splicing Assay—The *in vivo* splicing of the SMN2 minigene was carried out using a previously published procedure (35). Briefly, the SMN2 minigene was transfected into HEK293 cells with or without Myc-SRPK1 and varying amounts of EGFP-C2 vector containing the Tra2β1 cDNAs. Splice products were analyzed using RT-PCR.

Mass Spectrometry and RNA Binding Experiments—MALDI-TOF analyses were carried out using a Voyager DE-STR spectrometer. Tra2β1 constructs (1 μM) were incubated with SRPK1 (200 nM) and 0.3 mM ATP in 25 mM Mops (pH 7.2) and 10 mM free Mg^{2+} for 10 min or 2 h in a total volume of 100 μl. Reaction quenching and desalting were performed according to a prior method (34). The binding of RNA to the Tra2 proteins was measured using a nitrocellulose membrane and a Bio-Dot apparatus (Bio-Rad) according to a previously published protocol (36).

Single-turnover, Manual-mixing Experiments—Tra2 proteins (0.2 μM) were incubated with SRPK1 (1 μM) and $[^{32}P]ATP$ (100 μM) in a buffer containing 100 mM Mops (pH 7.4), 10 mM free Mg^{2+}, and 5 mg/ml BSA at 23 °C. All reactions were initiated with the addition of enzyme in a total reaction volume of 10 μl and quenched with SDS loading buffer. Phosphorylated protein was cut from an SDS-polyacrylamide gel (12%) and quantitated on the ^{32}P channel in liquid scintillant using previous methods (34).

Viscosity Experiments—Steady-state phosphorylation was monitored using a filter-binding assay in 0–30% sucrose according to previously published procedures (37). The relative solvent viscosity (η_{rel}) of the buffers (100 mM Mops (pH 7.4)) was measured using an Ostwald viscometer and a previously published protocol (38). η_{rel} values of 1.44, 1.83, 2.32, and 3.43 were measured for buffer containing 10, 20, 25, and 30% sucrose at 23 °C.

Rapid Quench Flow Experiments—Tra2 protein phosphorylation by SRPK1 was monitored using a model RGF-3 quench flow apparatus (KinTek Corp.). Typical experiments were performed by mixing equal volumes of the SRPK1-Tra2β1 complex in one reaction loop and $[^{32}P]ATP$ (5000–15,000 cpm/pmol) in the second reaction loop in 100 mM Mops (pH 7.4), 10 mM free Mg^{2+}, and 5 mg/ml BSA. All enzyme and ligand concentrations are those in the mixing chamber unless otherwise noted. The reactions were quenched with 30% acetic acid in the third syringe, and phosphorylated Tra2β1 was measured using a filter binding assay (37).

Data Analyses—The rate constants for several steps in the kinetic mechanism were extracted from viscosity dependences on the steady-state kinetic parameters according to Equations 1–3,

$$k_4 = \frac{k_{cat}}{(k_{cat})^\eta} \tag{Eq. 1}$$

$$k_p = \frac{k_{cat}}{(1 - (k_{cat})^\eta)} \tag{Eq. 2}$$

$$k_2 = \frac{k_{cat}/K_{SR}}{(k_{cat}/K_{SR})^\eta} \tag{Eq. 3}$$

where $(k_{cat})^\eta$ and $(k_{cat}/K_{SR})^\eta$ are the slopes of plots of the kinetic parameter in the absence and presence of viscosogen *versus* η^{rel} (38). In pre-steady-state kinetic experiments, the reaction product (P) as a function of time was fit to Equation 4,

$$[P] = \alpha(1 - \exp(-k_b t)) + k_L E_o t \tag{Eq. 4}$$

where α, k_b, k_L, and E_o are the amplitude of the "burst" phase, the rate constant for the burst phase, the rate constant for the linear phase, and the total enzyme concentration, respectively (39).

Results

SRPK1 Promotes Tra2β1-dependent Exon Inclusion in SMN2—Tra2β1 has been shown previously to enhance exon 7 inclusion in the *SMN2* gene in a concentration-dependent manner (28). Because SR and SR-like proteins are subject to phosphorylation-dependent regulation, we wished to determine whether SRPK1 could affect Tra2β1-dependent splicing. To accomplish this, we used a minigene in which an alternative exon is positioned by two constitutive exons in an SMN2 reporter construct (Fig. 1A). We expressed this reporter construct in the absence and presence of enhanced GFP-tagged Tra2β1 (EGFP-C2-Tra2β1) and Myc-tagged SRPK1 (Myc-SRPK1) vectors in HEK293 cells and monitored changes in alternative splicing by RT-PCR (30). We found that Tra2β1 increases exon inclusion in the SMN2 minigene in a concentration-dependent manner (Fig. 1B). This splicing change correlates with increases in Tra2β1 protein expression based on Western blotting (Fig. 1D). Importantly, the extent of exon 7 inclusion increases with SRPK1 co-expression (Fig. 1B). Tra2β1-induced SMN2 exon inclusion is abolished by expression of an inactive kinase (Myc-kdSRPK1) implying that catalytic activity is required for splicing function (Fig. 1C). These findings indicate that SRPK1 promotes inclusion of the alternative SMN2 exon 7 most likely by phosphorylating Tra2β1.

SRPK1 Phosphorylates Numerous Sites in Tra2β1—To understand how SRPK1 alters *SMN2* splicing, we investigated Tra2β1 phosphorylation by SRPK1. Tra2β1 contains two RS domains with 26 (RS1) and 8 (RS2) Arg-Ser dipeptides that are potential SRPK1 consensus sites (Fig. 2A). Whether all of these serines can be modified is not certain. We showed in a previous study that SRPK1 specifically phosphorylates the last 5 serines in a splice variant of Tra2β1 that lacks RS1 (Tra2β3) suggesting that the kinase may prefer to target serines in Arg-Ser stretches rather than isolated dipeptides (34). In this study we will refer to the splice variant Tra2β3 as Tra2β1(ΔRS1). To determine how many phosphates are added to Tra2β1, we expressed the full-length protein in *E. coli* and analyzed its purity and phosphorylation using a Phos-tag procedure (40). Tra2β1 phosphorylation by SRPK1 decreases mobility on Phos-tag SDS-PAGE (~160 kDa) compared with Tra2β1(ΔRS1) (~70 kDa) suggesting that more sites are modified in the full-length than the truncated substrate (Fig. 2B). Using MALDI-TOF, a lengthy 2-h incubation with SRPK1 increased the molecular mass of
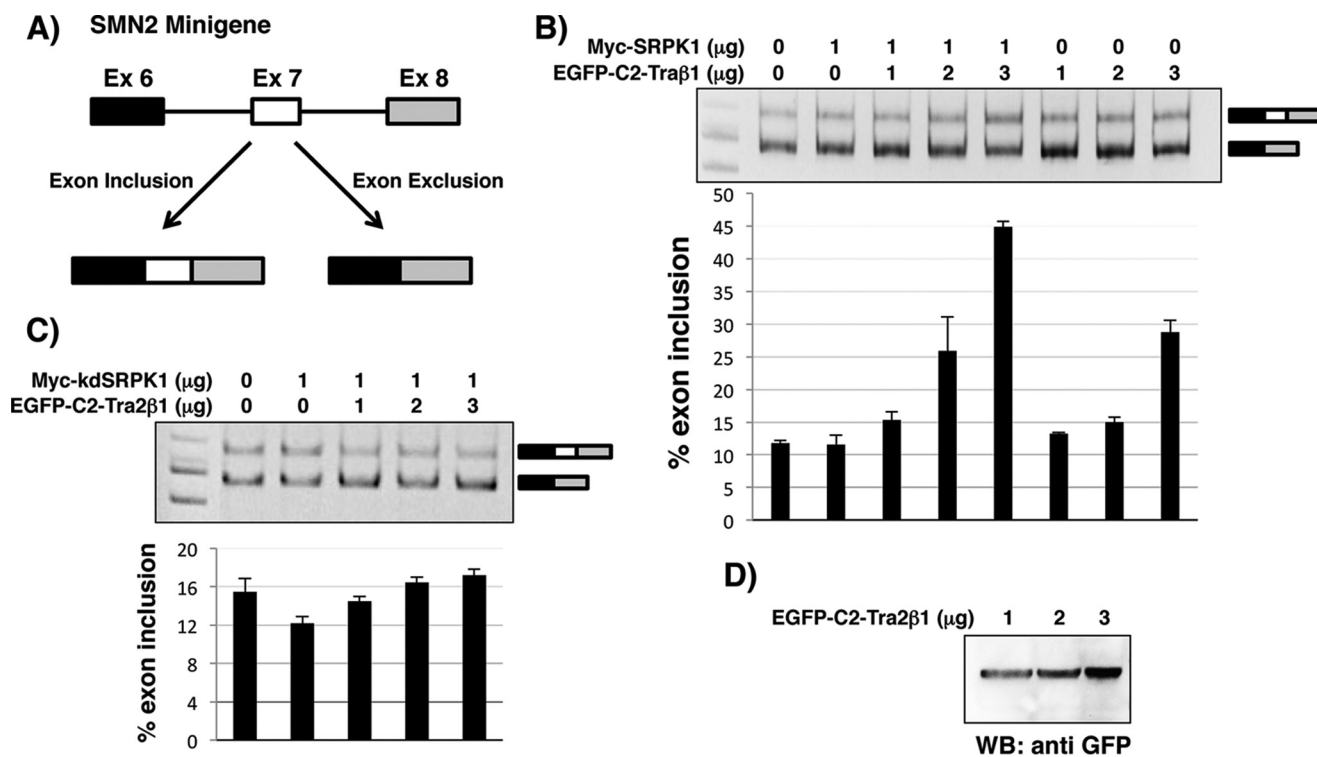
FIGURE 1. **SRPK1 induces Tra2β1-dependent splicing of SMN2.** *A,* SMN2 minigene. *B* and *C,* SMN2 minigene splicing as a function of Tra2β1 and SRPK1 (*B*) or kdSRPK1 (*C*) in HEK293 cells. *D,* Western blot (*WB*) detection of Tra2β1 in HEK293 cells.

Tra2β1 (35.1 to 37.1 kDa), corresponding to the addition of about 26 phosphates (Fig. 2*C*). To determine what subset of serines is rapidly phosphorylated and likely to be specific sites, we decreased the incubation time and found that SRPK1 added 21 phosphates in only 10 min (Fig. 2*D*). These phosphates are added rapidly with a half-life of ∼1 min in manual mixing, single-turnover experiments (Fig. 2*E*). Overall, these findings show that SRPK1 can rapidly and specifically modify 21 of the 34 potential phosphorylation sites in Tra2β1.

SRPK1 Uses a "Hit and Run" Strategy for Multisite Tra2β1 Phosphorylation—The SR protein SRSF1 stays physically associated with SRPK1 during multiple turnover events, whereas Tra2β1(ΔRS1) dissociates after each round of phosphorylation (40, 41). To determine whether the kinase uses a similar mechanism for Tra2β1, we performed a start-trap experiment in which kdSRPK1 is added to the reaction to trap any phospho-intermediates (42). In this experiment, ATP is added to the SRPK1-Tra2β1 complex with and without kdSRPK1 to monitor any changes in the reaction progress curve using the gel-based assay. If Tra2β1 does not dissociate from SRPK1 during multisite phosphorylation, kdSRPK1 will not affect the reaction when added simultaneously with ATP. However, if Tra2β1 dissociates then kdSRPK1 will trap the intermediates inhibiting the reaction. We found that the coordinate addition of kdSRPK1 and ATP to the active enzyme-substrate complex caused significant reductions in phosphorylation rate compared with the reaction lacking kdSRPK1 (Fig. 3*A*). In a trap-start control experiment, we pre-equilibrated the SRPK1-Tra2β1 complex with kdSRPK1 before the addition of ATP to make sure that the trap effectively inhibits the reaction. Pre-equilibration with kdSRPK1 lowered the initial velocity of the

progress curve from 6.5 to 0.3 sites/min (95% inhibition). Overall, we found that the start-trap and trap-start experiments were very similar suggesting that Tra2β1 is phosphorylated using a hit and run strategy where SRPK1 dissociates from the substrate after each round of catalysis.

Phosphoryl Transfer Step Limits Tra2β1 Turnover—To determine what limits Tra2β1 phosphorylation, we initially performed viscosometric experiments and found no changes in k_{cat} or k_{cat}/K_{SR} up to 30% sucrose, a relative viscosity increase of over 3-fold ($\eta^{rel} = 3.4$) (Fig. 3*B*). These findings can be understood using the mechanism in Scheme 1, where k_2 is the substrate association rate constant; k_p is the phosphoryl transfer rate constant, and k_4 is the net product release rate constant (ADP and phosphoprotein). We showed previously that these rate constants can be measured from the relative changes in kinetic parameters *versus* relative viscosity (η^{rel}) using Equations 1–3. Thus, in the absence of a viscosity effect on k_{cat} (*i.e.* $-(k_{cat})^{\eta} \sim 0$), k_p limits maximum turnover (0.36 s^{-1}), and k_4 is fast (>4 s^{-1}) (Table 2). The absence of a viscosity effect on k_{cat}/K_{SR} implies that the association rate constant for the protein is much larger than k_{cat}/K_{SR} and $k_2 > 40\ \mu M^{-1}\ s^{-1}$ (Table 2). To confirm these results, we showed that the production of phospho-Tra2β1 is linear in rapid quench flow experiments and shows no signs of a burst phase (Fig. 3*C*). Enzyme-normalized slopes close to k_{cat} (0.3 s^{-1}) were obtained, implying that the phosphoryl transfer step fully limits k_{cat}. Finally, to better define multisite phosphorylation, we performed a single turnover experiment in the rapid quench flow machine and showed that the first five phosphates are added in a time frame consistent with the steady-state and pre-steady state kinetic data (Fig. 3*D*). Interestingly, the phosphorylation rate per site declines
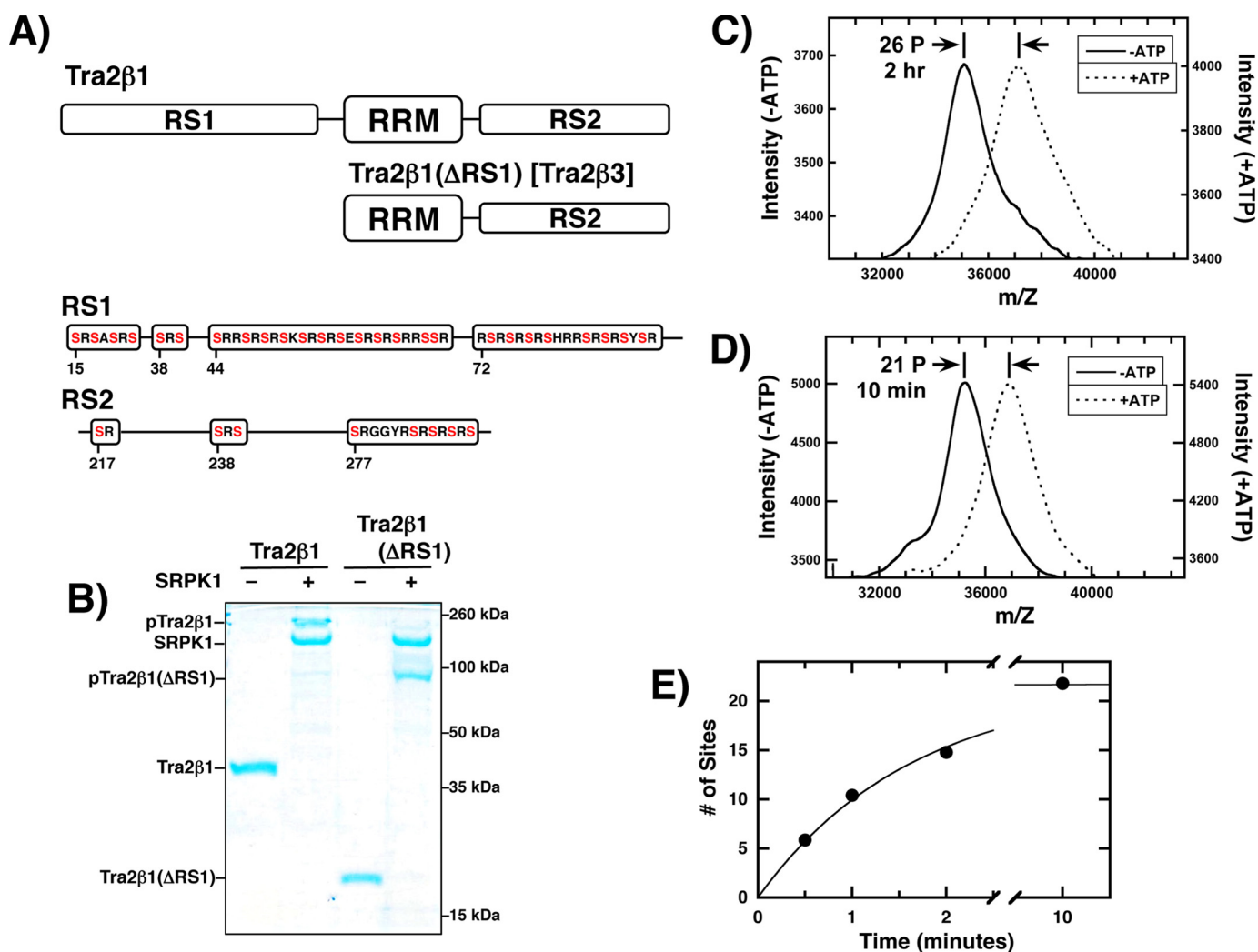
FIGURE 2. **Tra2β1 phosphorylation by SRPK1.** *A,* RS domains (RS1 and RS2) in Tra2β1 and Tra2β1(ΔRS1). *B,* Phostag SDS-PAGE of Tra2β1 and Tra2β1(ΔRS1) with and without SRPK1 phosphorylation. *C* and *D,* MALDI-TOF of Tra2β1 with SRPK1 after 10 min (*C*) and 2 h (*D*) of incubation with ATP. The molecular mass of Tra2β1 increases by 1.64 and 2.05 kDa after 10-min and 2-h incubations. *E,* SRPK1 phosphorylation of Tra2β1 by autoradiography. Data were fit to a rate constant of 0.6 min^{-1} and 21 total sites.

from 0.38 s^{-1} in the first five additions to 0.11 s^{-1} in the remaining additions suggesting that later phosphorylation events become slightly more difficult (Fig. 3*D*). Overall, the combined viscosometric and pre-steady-state kinetic data confirm that the phosphoryl transfer step limits Tra2β1 turnover.

RS1 Down-regulates RS2 Phosphorylation—Although we showed previously that SRPK1 modifies 5 serines in the splice variant Tra2β1(ΔRS1) (40), it is unclear whether these sites or any others in RS2 are also phosphorylated in the full-length Tra2β1. To address this, we initially removed a cluster of four possible phosphorylation sites in RS2 in Tra2β1(QM) (Fig. 4*A*). Based on Phos-tag SDS-PAGE, Tra2β1(QM) migrates similarly as Tra2β1 with and without SRPK1 treatment (Fig. 4*B*), suggesting that SRPK1 may phosphorylate the mutant substrate as well as Tra2β1. In MALDI-TOF experiments, Tra2β1(QM) and Tra2β1 were equally phosphorylated after a 10-min incubation with SRPK1 suggesting that RS2 is unlikely to be phosphorylated (Fig. 4*C*). To support this, we expressed a form of Tra2β1 lacking RS2 and all eight serines, Tra2β1(ΔRS2) (Fig. 4*A*), and we found that SRPK1 induced a large mobility shift

(~120 kDa; Fig. 4*D*). The magnitude of this shift is lower than that for Tra2β1, which could be the result of either a change in protein size and gel shift or a true decrease in overall phosphorylation. To address this, we measured the phosphoryl content of Tra2β1(ΔRS2) using MALDI-TOF and showed that after a 10-min or 2-h incubation with SRPK1, the molecular mass increased to a level consistent with about 21 or 27 phosphates (Fig. 4, *E* and *F*). These results are similar to those for the full-length substrate (Fig. 1) suggesting that SRPK1 phosphorylates RS1 rather than RS2 in Tra2β1. In summary, we conclude that RS1 not only is the major site for SRPK1 phosphorylation in Tra2β1 but also down-regulates phosphorylation in RS2.

RS2 Enhances ADP Release and Substrate Association Rates in SRPK1—Although RS2 plays no role in controlling the Tra2β1 phosphoryl content, we wished to determine whether it could affect the phosphorylation mechanism. In steady-state kinetic assays, we found that k_{cat} and k_{cat}/K_{SR} values for Tra2β1(ΔRS2) are about 3- and 9-fold lower than that for Tra2β1 (Table 1). To determine the cause of these decreases, we performed viscosity experiments. Whereas sucrose had
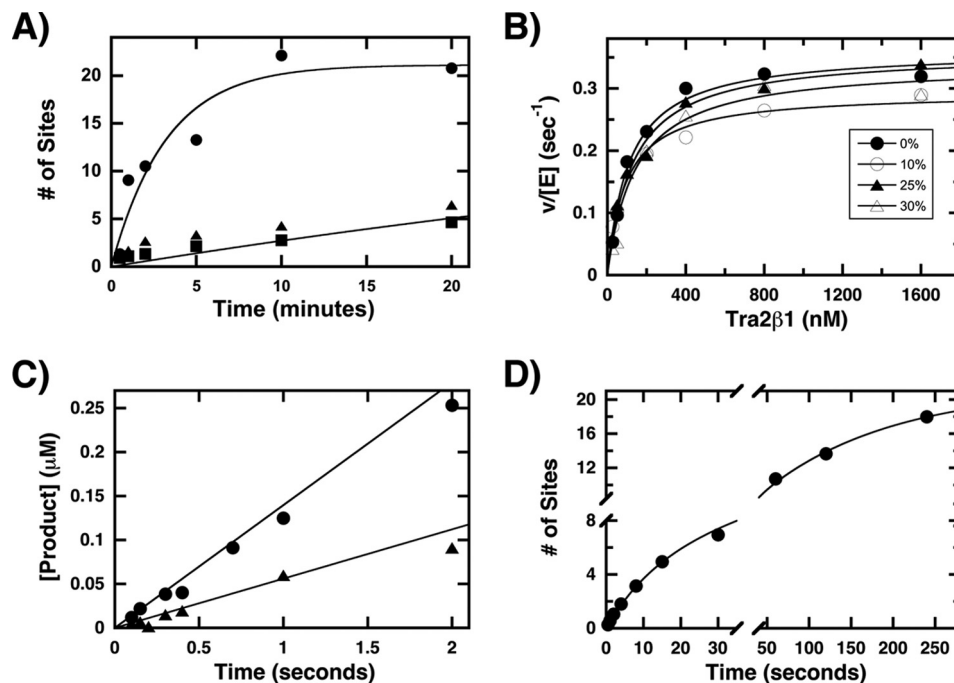
FIGURE 3. **Kinetic analysis of Tra2β1.** *A,* start-trap experiment. SRPK1 (1 μM) and Tra2β1 (0.2 μM) are pre-equilibrated before addition of ATP (50 μM) in the absence (●) and presence (▲) of kdSRPK1 (60 μM). The data in the absence of kdSRPK1 are fit to a rate constant of 0.31 min^{-1} and an end point of 21 sites. A control reaction is run by pre-equilibrating SRPK1, kdSRPK1, and Tra2β1 prior to ATP addition (■) and fit to a slope of 0.29 sites/min. *B,* viscosity experiments. Initial velocity of SRPK1 is measured as a function of Tra2β1 at varying amounts of sucrose. The data at 0% sucrose are displayed in Table 1. *C,* rapid quench flow experiments. Tra2β1 (3 μM) is pre-equilibrated with 0.2 (▲) and 0.5 μM (●) SRPK1 and then reacted with 50 μM ATP. The data are fit to slopes of 0.06 and 0.14 μM/s at 0.2 and 0.5 μM SRPK1. *D,* single turnover experiment. SRPK1 (2 μM), Tra2β1 (0.4 μM), and ATP (100 μM) are reacted in the rapid quench flow instrument, and the number of sites are plotted with time (0.1–240 s). The data are fit to a double exponential with amplitudes and rate constants of 5.0 and 16 sites and 0.075 and 0.0065 s^{-1}.
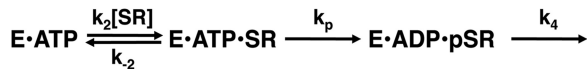
SCHEME 1

no effect on Tra2β1, both k_{cat} and k_{cat}/K_{SR} values for Tra2β1(ΔRS2) were significantly reduced (Fig. 5*A*). Ratio plots of k_{cat} and k_{cat}/K_{SR} in the absence and presence of sucrose (relative parameter) *versus* relative viscosity (η^{rel}) are linear with slopes close to the theoretical upper limit of 1 (Fig. 5*B*). This suggests that, unlike the full-length substrate, Tra2β1(ΔRS2) phosphorylation is limited by net product release (k_4) (Table 2). Using Equation 3 and the viscosity effects on k_{cat}/K_{SR}, we show that this parameter directly measures the substrate association rate constant (k_2) (Table 2). These findings indicate that RS2 increases the substrate association rate to SRPK1 by about 2 orders of magnitude (Table 2). To confirm that the phosphoryl transfer step is not rate-limiting for k_{cat}, we performed pre-steady-state kinetic experiments. We found that Tra2β1(ΔRS2), unlike the full-length substrate, is phosphorylated in a biphasic manner with an initial burst phase ($k_b = 1$ s^{-1}) followed by a linear steady-state phase ($k_L = 0.11$ s^{-1}) (Fig. 5*C*). The presence of a burst indicates that the phosphoryl transfer step for Tra2β1(ΔRS2) is fast and does not limit k_{cat}. Overall, these data show that the addition of RS2 increases substrate association and net product release rates.

To isolate the rate-limiting step for Tra2β1(ΔRS2) phosphorylation, we performed a catalytic trapping experiment in which the SRPK1-Tra2β1(ΔRS2) complex is pre-equilibrated with ADP before mixing with excess [^{32}P]ATP in the rapid quench

flow machine (43). Without ADP pre-equilibration, we observed burst kinetics that were simulated using the mechanism in Scheme 2 and the program DynaFit (Fig. 5*D*) (44). Using a fixed value for the ATP k_{on} (0.013 μM^{-1} s^{-1}) based on k_{cat}/K_{ATP}, we obtained values of 0.45 and 0.11 s^{-1} for k_p and k_4 (Table 2). The latter reflects the net rate constant for product release, and its value is consistent with k_{cat} (Table 1). Pre-equilibration of the complex with ADP leads to a loss of the burst phase and the generation of a small lag prior to resumption of steady-state turnover (Fig. 5*D*). The linear phases with and without ADP are similar indicating that sufficient ATP traps any free complex. The data in the presence of ADP were simulated using the mechanism in Scheme 2. By fixing k_{on}, k_p, and k_4, we obtained a value of 0.16 s^{-1} for the ADP k_{off} (k_{ADP}). These findings indicate that maximum turnover of Tra2β1(ΔRS2) is mostly limited by ADP release. By considering the simulated values of k_p (0.45 s^{-1}) and k_{ADP} (0.16 s^{-1}), we can calculate a lower limit for the release rate of phospho-product (k_{pSR}) of 2 s^{-1} (Table 2). By comparing Tra2β1 and Tra2β1(ΔRS2), we conclude that RS2 enhances overall RS domain phosphorylation by increasing k_{ADP} from 0.16 to >4 s^{-1} and by increasing k_2 from 0.41 to >40 μM^{-1} s^{-1} (Table 2). Despite these changes, RS2 has no detectable effect on the phosphoryl transfer rate.

Efficient RS1 Phosphorylation Requires the Docking Groove— In previous studies we showed that although efficient phosphorylation of SRSF1 requires the docking groove in SRPK1, the shorter repeat in Tra2β1(ΔRS1) does not (40). We wished to learn whether RS1 phosphorylation in the full-length Tra2β1 also requires this docking groove. We initially confirmed that
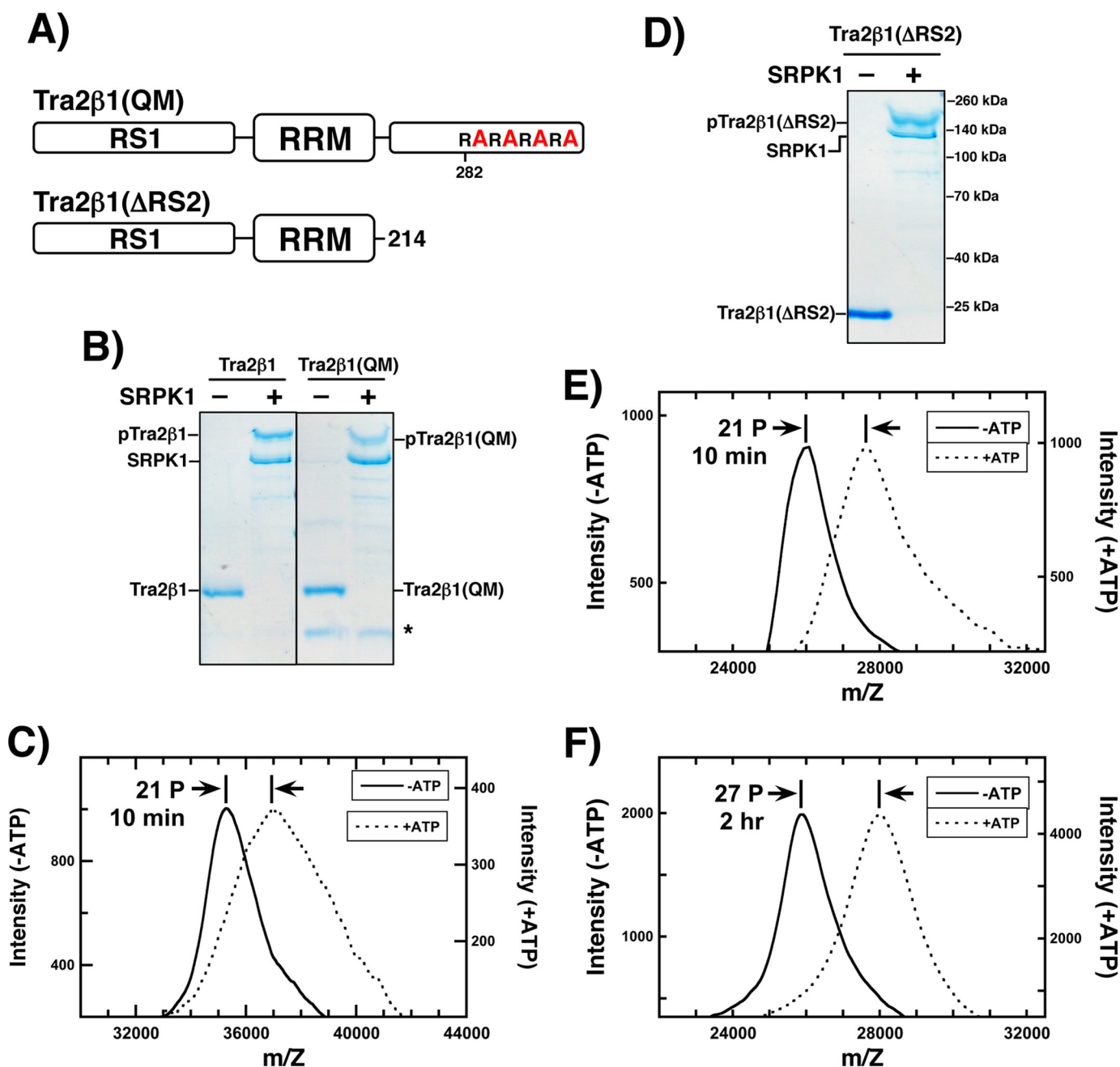
FIGURE 4. **Mutation of phosphorylation sites in RS2.** *A*, Ser-to-Ala mutations and deletion constructs of RS2. *B*, Phos-tag SDS-PAGE analysis of Tra2β1(QM) and Tra2β1 with and without SRPK1 phosphorylation. *C*, MALDI-TOF of Tra2β1(QM) after a 10-min incubation with SRPK1 and ATP. The molecular mass of Tra2β1(QM) increases by 1.65 kDa (21 phosphates). *D*, Phos-tag SDS-PAGE analysis of Tra2β1(ΔRS2) with and without SRPK1 phosphorylation. *E* and *F*, MALDI-TOF of Tra2β1(ΔRS2) after 10-min (*E*) and 2-h (*F*) incubation with SRPK1 and ATP. The molecular mass of Tra2β1(ΔRS2) increases by 1.64 and 2.11 kDa after 10-min and 2-h incubations.

TABLE 1
Steady-state kinetic parameters for the phosphorylation of wild type and mutant Tra2β1 proteins

Substrate	k_{cat}	K_{SR}	k_{cat}/K_{SR}	K_{ATP}	k_{cat}/K_{ATP}
	s^{-1}	nM	$\mu M^{-1} s^{-1}$	μM	$\mu M^{-1} s^{-1}$
Tra2β1	0.36 ± 0.02	110 ± 16	3.6 ± 0.53	10 ± 2	40 ± 8.1
Tra2β1(ΔRS2)	0.11 ± 0.01	270 ± 60	$0.41 \pm .10$	9 ± 4	12 ± 5.4
Tra2β1(ΔRS1)	0.032^a	35^a	0.91^a	11^a	2.9^a

[a] Data were reported previously (34).

Tra2β1(ΔRS1) does not require this groove, as reported previously (40), by showing that phosphorylation is not diminished by a mutant SRPK1, SRPK1(6M), that lacks a functioning dock-

ing groove (Fig. 6*A*). SRPK1(6M) contains alanine mutations in six electronegative residues in the docking groove of the kinase domain (33). In contrast to Tra2β1(ΔRS1), we found that the
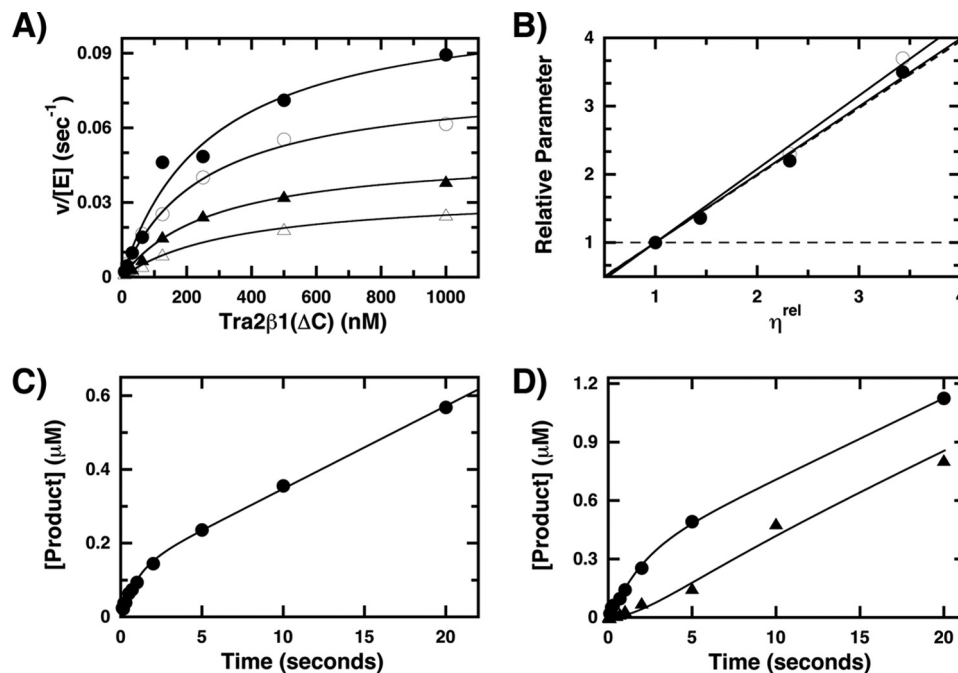
FIGURE 5. **Kinetic analysis of Tra2β1(ΔRS2).** *A,* viscosity experiments. Initial velocity of SRPK1 is measured as a function of Tra2β1(ΔRS2) at varying sucrose amounts. The data fits at 0% sucrose are displayed in Table 1. *B,* viscosity plot. The relative parameters for k_{cat} (●) and k_{cat}/K_{SR} (○) in the absence and presence of viscosogen are plotted as a function of relative viscosity (η^{rel}). Theoretical slope values of 0 and 1 are shown as *dashed lines*. *C,* rapid quench flow experiments. Tra2β1(ΔRS2) (1 μM) is pre-equilibrated with 0.2 μM SRPK1 before 50 μM ATP addition. The data are fit to Equation 4 to obtain values of 0.12 ± 0.01 μM, 1.0 ± 0.2 s^{-1}, and 0.11 ± 0.04 s^{-1} for α, k_b, and k_L, respectively. *D,* catalytic trapping ($C_{AT}T_{RAP}$) experiments. SRPK1 (0.4 μM) and Tra2β1(ΔRS2) (1 μM) are pre-equilibrated with (▲) and without (●) ADP (60 μM) before ATP addition (600 μM). The data are simulated using the mechanism in Scheme 2 to obtain values of 0.45, 0.11, and 0.16 s^{-1} for k_p, k_4, and k_{ADP}, respectively (Table 2).

TABLE 2

Individual rate constants associated with the phosphorylation of wild type and mutant Tra2β1 proteins

Rate constant	Tra2β1	Tra2β1(ΔRS2)	Tra2β1(ΔRS1)
k_2 (μM^{-1}s^{-1})	≥40[a]	0.41[a]	1.1[b]
k_p (s^{-1})	0.30[c] (0.36[a])	0.45[c]	0.23[b]
k_4 (s^{-1})	≥4[a]	0.11[a,c]	0.028[b]
k_{ADP} (s^{-1})	>4[a]	0.16[c]	0.34[b]
$k_{p,\,SR}$ (s^{-1})	>4[a]	>2[a]	0.030[b]

[a] Data were determined using viscosometric experiments and Equations 1–3.
[b] Data were previously reported (34).
[c] Data were determined using rapid quench flow experiments.

SCHEME 2
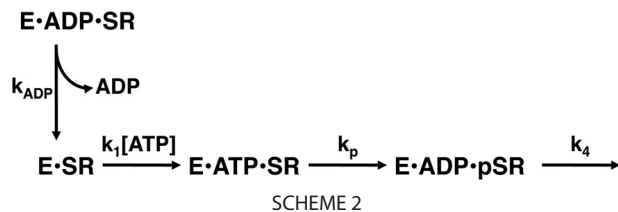
rate for Tra2β1 was much lower for SRPK1(6M) compared with the wild-type kinase over the assay time course suggesting that the lengthy repeats in RS1 require the docking groove (Fig. 6*B*). Tra2β1(ΔRS2) also requires the docking groove for rapid multisite phosphorylation (Fig. 6*C*). Overall, these findings suggest that the docking groove plays an important role in regulating the efficiency of RS1 phosphorylation and that RS2, although not phosphorylated, likely plays a role in positioning RS1 in this groove.

RS2 Enhances RNA Binding to Tra2β1—Having shown that RS2 augments the rate of Tra2β1 phosphorylation by SRPK1, we next wondered whether this C-terminal RS domain could

also impact RRM function. To accomplish this, we monitored the interaction of Tra2β1 and the two RS domain deletion constructs with an RNA sequence based on the consensus ESE for Tra2β1 (AAGAAC) using a filter binding assay (22). We found that Tra2β1 along with Tra2β1(ΔRS1) and Tra2β1(ΔRS2) bind cooperatively to the ESE (Fig. 7*A* and Table 3). Because of the small length of the RNA strand, the observed cooperativity is likely the result of protein-protein interactions that enhance RNA association with the RRM. Although the three proteins showed similar Hill coefficients, they displayed differences in overall binding affinities ($K_{0.5}$). Whereas Tra2β1(ΔRS1) binds with similar affinity as the full-length protein, Tra2β1(ΔRS2) displayed reduced binding affinity (Fig. 7*A* and Table 3). Because of the high level of cooperativity, this 2-fold change in binding has significant impact on the bound states. For example, at concentrations near $K_{0.5}$ (8 μM), 10 times more Tra2β1 is bound to RNA compared with Tra2β1(ΔRS2) (Fig. 7*A*). These findings suggest that the ability of the RRM to associate with RNA is enhanced by the presence of RS2. To determine whether the RS domain could bind in the absence of the RRM, we expressed and purified a His-tagged form of RS2. We found essentially no binding up to 14 μM RS2, the highest concentration achievable for this construct, whereas 90% of the RNA is bound at a similar concentration of Tra2β1(ΔRS1) or Tra2β1 (Fig. 7*A*). These results indicate that RS2 does not interact directly with RNA but instead induces a higher affinity form of the RRM.

Phosphorylation Dissociates the Tra2β1-RNA Complex—To ascertain whether phosphorylation alters RNA binding, we pre-formed RNA-protein complexes and asked whether
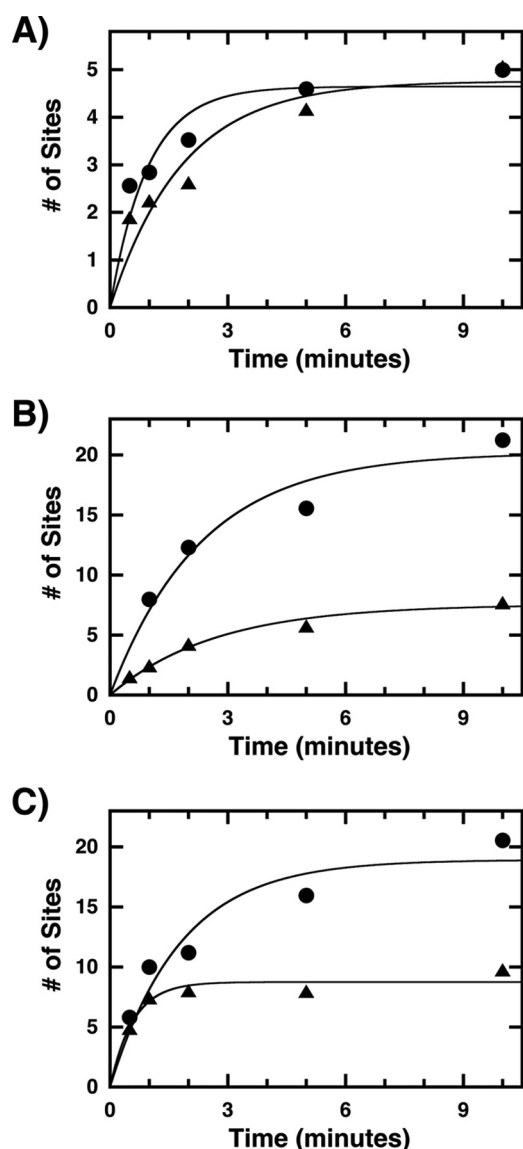
FIGURE 6. **Role of docking groove for substrate phosphorylation.** ATP (100 μM) and 1 μM SRPK1 (●) or SRPK1(6M) (▲) are reacted with 0.2 μM Tra2β1(ΔRS2) (*A*), Tra2β1 (*B*), or Tra2β1(ΔRS2) (*C*).

FIGURE 7. **Interactions of *SMN* ESE with wild-type and mutant forms of Tra2β1.** *A*, binding profile. ESE from *SMN* is equilibrated with Tra2β1, Tra2β1(ΔRS1), and Tra2β1(ΔRS2) and the fraction bound is plotted against varying protein concentrations. Parameter fits for the data are displayed in Table 3. *B*, change in fractional bound RNA as a function of phosphorylation. ESE from SMN is equilibrated with fixed amounts of Tra2β1 proteins at their $K_{0.5}$ values (Table 3) and then treated with SRPK1, ATP, or SRPK/ATP. The change in fractional bound RNA is displayed relative to the ESE-protein complex in the absence of any additional agents.

TABLE 3

Binding of RNA to mutant and wild type Tra2β1 proteins

Protein	$K_{0.5}$	N
	μM	
Tra2β1	7.7 ± 0.32	3.3 ± 0.44
Tra2β1(ΔRS1)	8.0 ± 0.16	3.1 ± 0.20
Tra2β1(ΔRS2)	18 ± 0.35	3.7 ± 0.20

SRPK1 phosphorylation affects their bound states. For these experiments, we fixed the total protein concentrations near $K_{0.5}$ so that we could sensitively measure small increases/decreases in phosphorylation-dependent RNA binding. We found that SRPK1 phosphorylation of all three proteins significantly reduced RNA binding (Fig. 7*B*). To ensure that this reduction is not due to a secondary binding event, we showed that the addition of SRPK1 or ATP to the protein-RNA complex did not induce dissociation (Fig. 7*B*). Assuming similar levels of binding cooperativity, these findings suggest that SRPK1 phosphorylation reduces RNA affinity by ~2-fold to Tra2β1(ΔRS1) and Tra2β1(ΔRS2) and 4-fold to Tra2β1. These results demonstrate that the two RS domains serve opposing, phosphorylation-dependent roles with regard to RNA binding. Whereas RS2 supports RNA binding in unphosphorylated Tra2β1, RS1 inhibits binding in phosphorylated Tra2β1.
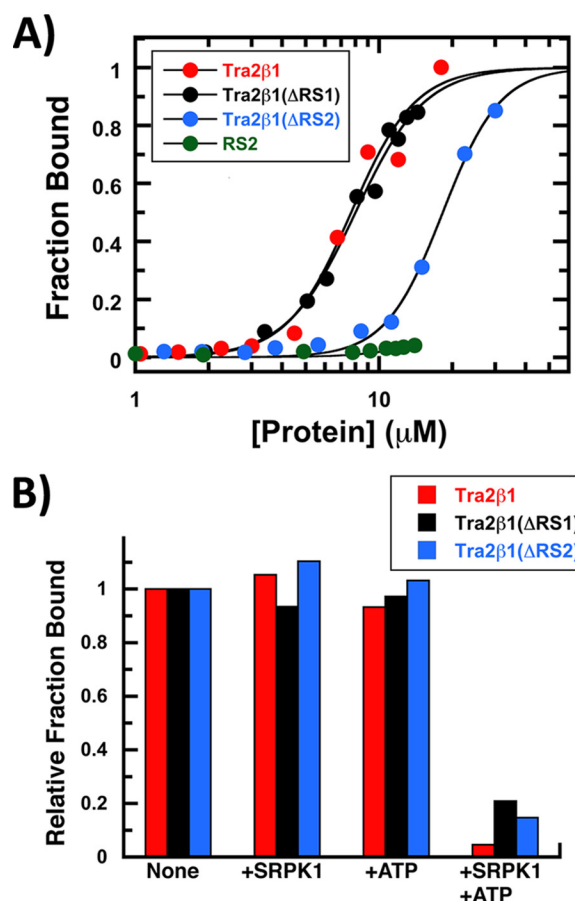
SRPK1 Activation of Tra2β1-dependent SMN2 Splicing Requires RS1 and RS2—Previous studies demonstrated that removal of either RS1 or RS2 reduces exon 7 inclusion in the SMN2 minigene by about 30% suggesting that both RS domains are required for efficient Tra2β1-dependent splicing (45). We wished to address whether both RS domains are required for SRPK1-dependent splicing activation. We found that SRPK1 expression did not increase exon 7 inclusion in the SMN2 minigene when co-expressed with either Tra2β1(ΔRS1) or Tra2β1(ΔRS2) (Fig. 8). Deletion of either RS1 or RS2 lowered exon 7 inclusion by 20–40% indicating that SRPK1 does not rescue this phenotype. These findings suggest that both RS domains are required for SRPK1-dependent activation of Tra2β1-induced splicing.
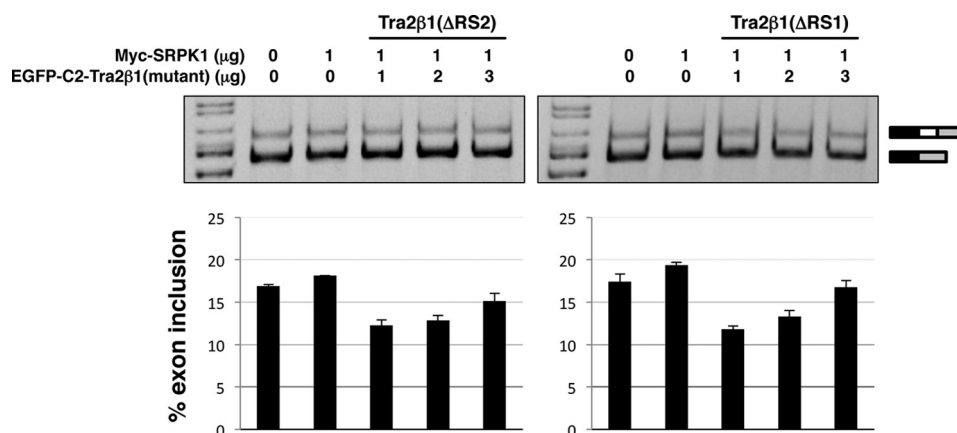
FIGURE 8. **Role of RS1 and RS2 for SRPK1-dependent splicing of SMN2.** SMN2 splicing is monitored in the presence of Tra2β1(ΔRS1) or Tra2β1(ΔRS2) and SRPK1 expression in HEK293 cells.
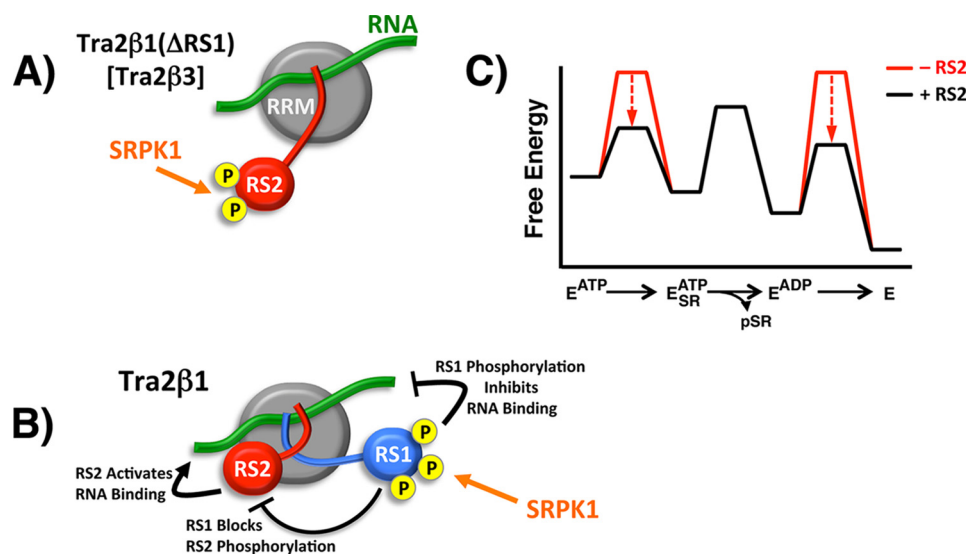

FIGURE 9. **Effects of RS domain on Tra2β1 and SRPK1 function.** *A,* RS2 disengages from the RRM in Tra2β1(ΔRS1). *B,* coordinate roles of RS1 and RS2 on phosphorylation and RNA binding in Tra2β1. *C,* RS2 accelerates Tra2β1 phosphorylation by enhancing protein substrate association and product release rates. Free energy profile compares relative changes for Tra2β1 (*black*) and Tra2β1(ΔRS2) (*red*).

Discussion

Detailed studies of the prototype SR protein SRSF1 suggest that SRPKs target lengthy Arg-Ser repeats phosphorylating them in a C-to-N directional manner (15). The efficiency of this process is guided by a docking groove in the kinase domain of SRPK1 that feeds Arg-Ser dipeptides to the active site. In this work we address how SRPK1 phosphorylates Tra2β1, an SR-like protein that contains two RS domains and controls gene splicing in a phosphorylation-dependent manner (10, 46). Interestingly, although most SR proteins gain entry into the nucleus through a phosphorylation-dependent process, hyper-phosphorylation drives Tra2β1 into the cytoplasm (30, 31), similar to that for the splicing regulator hnRNP1 (47). We show for the first time that SRPK1 directly phosphorylates Tra2β1, a modification that regulates the alternative splicing of the *SMN2* gene. Because of the role of this gene in spinal muscle atrophy, SRPK1 expression and its Tra2β1-dependent phosphorylation activity could serve a positive role in tipping the balance from truncated to full-length SMN proteins, leading to possible therapeutic avenues for treating this disease. We show that the two

RS domains in Tra2β1 engage in extensive cross-talk that regulates not only protein phosphorylation of the domains but also RNA binding and ligand exchange events in SRPK1.

Eliminating the Competition by Changing Strategies—Although we showed in a previous study that RS2 in the splice variant Tra2β1(ΔRS1) is a good substrate for SRPK1 (34), the introduction of RS1 in the full-length splicing factor profoundly affects access to RS2. The switch from RS2 to RS1 phosphorylation in Tra2β1 is surprising for two reasons. First, Tra2β1(ΔRS1) binds with 4-fold higher affinity than the full-length Tra2β1 to SRPK1 (Table 1) suggesting the formation of a highly productive enzyme-RS2 complex. Second, the phosphoryl transfer rate in the active site of SRPK1 (k_p) is the same for Tra2β1 and Tra2β1(ΔRS1) (Table 1), an observation suggesting that RS2 is well aligned for efficient in-line transfer. If we compare the catalytic efficiencies of SRPK1 for Tra2β1(ΔRS1) and Tra2β1(ΔRS2) using k_{cat}/K_{SR} as a metric (Table 1), we would anticipate that RS2 is more likely to be phosphorylated than RS1, the opposite of our findings. To explain this paradox, we propose that the two RS domains interact differently

with the central RRM (Fig. 9). In the splice variant lacking RS1 (Tra2β1(ΔRS1)), the RRM may be weakly associated with the full RS2 segment such that SRPK1 can readily phosphorylate serines on the C-terminal end explaining the efficient kinetic parameters for Tra2β1(ΔRS1) (Fig. 9A). However, RS1 may induce a conformational change that imposes closer ties between RS2 and the RRM, thus, limiting kinase access (Fig. 9B). This mechanism is supported by the RNA binding assays that show that RS2 removal in Tra2β1(ΔRS2) weakens interactions with an ESE compared with the RS2-containing substrates Tra2β1 and Tra2β1(ΔRS1) (Table 3). By coupling RS2 with the RRM, Tra2β1 offers unequal access to the two RS domains. Thus, RS1 may not behave as a free competitor but instead may alter the conformation of the protein to eliminate its competition.

Embedding a Phosphorylation-dependent Switch in Tra2β1—Phosphorylation is important for the regulation of many splicing factors from both the SR and SR-like families. SR proteins have been shown to undergo rounds of phosphorylation and dephosphorylation during spliceosome assembly (4, 48). Furthermore, localization of these factors is controlled by phosphorylation-dependent interactions with a specific transportin (TRN-SR) that promotes entry through the nuclear pore and deposition in nuclear speckles (18, 49). In the case of the SR-like Tra2β1, the alternative splicing of several genes has been linked to both hypo- and hyper-phosphorylation levels (29, 46, 50). Although the details of these processes are not well understood at a molecular level, it is anticipated that large phosphorylation-dependent structural changes in these splicing factors may be required for any biological regulatory mechanism. Previous studies showed a connection between RNA binding and RS domain phosphorylation. The RRM of Tra2β1 harbors a protein phosphatase 1 (PP1)-binding site, which facilitates dephosphorylation of Tra2β1. Inhibition of PP1 promotes hyper-phosphorylation and *SMN2* exon 7 inclusion (46), similar to SRPK1 overexpression (Fig. 1B). In our studies, we now present a mechanism whereby the two RS domains provide a means for both up- and down-regulation of RNA binding (Fig. 9B). We show that the central RRM in Tra2β1 is not an efficient binder of RNA unless RS2 is present, suggesting close ties between the RRM and RS2. Furthermore, these interactions could position RS2 for favorable electrostatic contacts with RNA. Although RS1 does not modulate RNA binding in its unphosphorylated state, SRPK1 phosphorylation of RS1 significantly impairs interactions of the RRM with the ESE. This could result from electrostatic repulsion between the phospho-RS1 and ESE and/or repulsion of the two RS domains that move toward each other after RNA binding (45). Thus, the two RS domains and their phosphorylation states regulate RNA interactions. Changes in RNA binding affinity are likely necessary during the splicing reaction that entails multiple changes in RNA interactions. We propose that the phosphorylation-dependent affinity switch of Tra2β1 fine-tunes the affinity of the protein and the pre-mRNA.

RS Domain Regulates Ligand Exchange in SRPK1—In addition to important internal contacts within Tra2β1, we found that the RS domains can also regulate the catalytic efficiency of SRPK1. In particular, the observation that RS2 raises both k_{cat}

and k_{cat}/K_{SR} implies that the C-terminal RS domain is a positive regulator of SRPK1 irrespective of any changes in the cellular levels of Tra2β1. The underlying causes of this concentration-independent enhancement are best described in a reaction-free energy profile (Fig. 9C). Although RS2 has no effect on the phosphoryl transfer rate, it greatly increases the rates of two ligand exchange events, substrate binding and product release. We showed that RS2 amplifies the association rate constant for Tra2β1 by at least 100-fold and increases ADP release by about 20-fold (Table 2). The former result could be due to an inductive effect across the substrate that helps align RS1 for productive encounters with SRPK1. Also, it is possible that the RRM-RS2 pair makes direct contacts with SRPK1 assisting RS1 into the active site. The latter mechanism may also provide a means for assisting ADP release. In previous studies we showed that the N-terminal extension and a helix in an insert domain in SRPK1 constitute a nucleotide release factor that accelerates ADP dissociation through interactions with a portion of the RS domain in SRSF1 (51, 52). Using this precedent, it is possible that the RRM-RS2 pair in Tra2β1 interacts with this factor accelerating ADP release from SRPK1. Our studies on the atypical splicing factor Tra2β1 now show that phosphorylation can also be accelerated by secondary RS domains distal from the primary RS domain target.

Conclusions—In this study we demonstrate for the first time that SRPK1 can phosphorylate Tra2β1 and induce exon inclusion in the *SMN2* gene. The phosphorylation mechanism for Tra2β1 departs from the classic paradigm established for the prototype SR protein SRSF1. The two RS domains (RS1 and RS2) act in a synergistic manner not only to regulate the availability of serines for phosphorylation but also to manipulate the kinetic mechanism of SRPK1 (Fig. 9). Although both RS domains contain consensus serines for SRPK1, RS1 down-regulates phosphorylation of the RS2 serines. Both domains play phosphorylation-dependent and -independent functions in RNA binding. The unphosphorylatable RS2 domain enhances RNA binding to RRM, whereas the phosphorylated RS1 domain diminishes this activity. The phosphorylation-dependent release of RNA could then facilitate transport of Tra2β1 to the cytoplasm. Although not a target for SRPK1, RS2 can enhance the interactions of RS1 with the active site of SRPK1 and promote efficient substrate turnover by increasing product dissociation. The cooperativity between RS1 and RS2 is essential for the SRPK1-dependent effects on *SMN2* splicing. These findings significantly broaden our understanding of how RS domains in this splicing factor are phosphorylated and how Arg-Ser repeats widely separated in sequence can cross-regulate function.

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