

Protein Kinase A–Dependent Phosphorylation of Serine 119 in the Proto-Oncogenic Serine/Arginine-Rich Splicing Factor 1 Modulates Its Activity as a Splicing Enhancer Protein

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Anne Kristin Aksaas¹, Sissel Eikvar², Göran Akusjärvi³, Bjørn S. Skålhegg¹, and Anne Katrine Kvissel^{1,2}

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Abstract

Serine/arginine-rich splicing factor 1 (SRSF1), previously designated SF2/ASF, belongs to a family of SR proteins that regulate constitutive and alternative splicing. SRSF1 expression is increased in tumors from several tissues and elicits changes in key target genes involved in tumor genesis. Several protein kinases phosphorylate SRSF1, which regulates its localization and function. It is previously reported that protein kinase A (PKA) phosphorylates SRSF1, but the importance of this modification is not well characterized. Here, we show that PKA phosphorylates SRSF1 on serine 119 *in vitro*. Phosphorylation of SRSF1 on this site enhanced the RNA binding capacity of SRSF1 *in vivo* and reduced the protein's capacity to activate splicing of the Minx transcript *in vitro*. We also confirm an interaction between SRSF1 and PKA C α 1 and demonstrate that this interaction is not dependent on serine 119 phosphorylation but requires active PKA C α 1. We conclude that PKA phosphorylation of SRSF1 at serine 119 regulates SRSF1-dependent RNA binding and processing but not its interaction with PKA.

Keywords

pre-mRNA splicing regulation, SRSF1, PKA, phosphorylation

Introduction

Gene expression in eukaryotic cells is regulated at several levels and by numerous mechanisms. Transcription of protein encoding genes results in the synthesis of a precursor messenger RNA (pre-mRNA), which contains a sequence of coding and noncoding RNA in addition to a 5' cap and a 3' polyadenine tail. The coding exons are joined together by a process called pre-mRNA splicing to produce the mature RNA that is exported to the cytoplasm and translated into protein. This process is catalyzed by the spliceosome, which consists of large RNA protein complexes that assemble in a stepwise manner onto the pre-mRNA.^{1,2} Alternative splicing is an important mechanism to increase the proteome complexity by allowing production of structurally and functionally distinct protein isoforms from a single transcript. Estimates predict that as much as 92% to 94% of the human genes can produce alternatively spliced mRNAs, and this can be achieved by exon skipping, intron retention, mutual exclusion of exons, alternative first or last exon, or use of alternative 3' and 5' splice sites.³ Alternatively spliced isoforms are commonly found during development and in tissue-specific expression.⁴ In tumor biology, constitutively expressed proteins often possess control functions in the cell, such as growth and programmed cell death, while

alternatively spliced isoforms tend to promote uncontrolled cell growth, invasive properties, and resistance towards apoptosis. These tumor-promoting behaviors are confirmed by the fact that these isoforms tend to be overexpressed in many cancer cells and tissues.^{5,6} Splice-site selection under normal physiological conditions is highly regulated, and errors resulting in aberrant transcripts encoding truncated proteins rarely occur. In contrast to this, errors in splicing events seem to be an intrinsic property of cancer cells.^{7,8}

The pre-mRNA splicing process is regulated by both internal and external factors. Exonic and intronic splicing enhancers and silencers are RNA sequences that interact with external splicing factors, which again can promote or suppress the spliceosome assembly.⁹ Human serine/arginine-rich proteins (SR proteins) are a family of 12

¹Department of Nutrition, University of Oslo, Oslo, Norway

²Department of Biochemistry, University of Oslo, Oslo, Norway

³Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden

Corresponding Author:

Bjørn S. Skålhegg, Department of Nutrition, University of Oslo, Sognsvannsveien 9, Oslo, Pb 1046 Blindern, Norway
Email: b.s.skålhegg@medisin.uio.no

splicing factors characterized by 1 or 2 N-terminal RNA binding domains (RBDs) followed by a domain rich in arginine and serine dipeptide repeats (RS domain) of a minimum 50 amino acids.¹⁰ In general, SR proteins interact with exonic splicing enhancer elements (ESEs) and promote binding of the spliceosomal elements U2AF and/or U1 snRNP to splice sites.² In addition to being essential in pre-mRNA splicing, SR proteins are implicated in mRNA nuclear export, mRNA stability, nonsense-mediated mRNA decay (NMD), translation, and oncogenic transformation.¹¹⁻¹⁵ SR proteins are extensively phosphorylated *in vivo*, mainly in the RS domain, a modification that plays an important role in regulating their subcellular localization and activity.¹³ During recent years, it has become increasingly evident that molecular changes mediated by signal transduction pathways control splice-site selection *in vivo*.¹⁶ Reversible phosphorylation cascades are able to conduct rapid signals throughout the cell and are probably important in mediating extracellular signals to the spliceosome.¹⁷

Serine/arginine-rich splicing factor 1 (SRSF1), previously designated alternative splicing factor/splicing factor 2 (SF2/ASF),¹⁰ is a prototypical SR protein. It is involved in both constitutive and alternative splicing and contains 2 RBDs and a C-terminal RS domain.¹⁸ Moreover, SRSF1 has been reported to be a proto-oncogene whose increased expression in tumors from several tissues elicits changes in key target genes involved in tumor genesis.¹⁹ In addition, SRSF1 is located to a genomic region, which is amplified in several cancers and is correlated with poor prognosis.²⁰ SRSF1 has also been shown to promote tumor transformation and growth by several mechanisms, for example, by stabilizing mRNA of antiapoptotic factors²¹ and by generating inactive tumor suppressor proteins by alternative splicing.^{19,22} SRSF1's activity and function are regulated by phosphorylation by several protein kinases, such as the SR-specific protein kinase 1 (SRPK1),²³ Cdc2-like kinases (Clk/Sty),²⁴ topoisomerase I,²⁵ and dual-specificity tyrosine phosphorylation-regulated kinase 1A (Dyrk1A).²⁶ Phosphorylation by SRPK1/2 and Clk/Sty modulates shuttling of SRSF1s between the cytoplasm, nucleus, nuclear speckles, and active transcription sites.¹⁷ Cyclic AMP (cAMP)-dependent protein kinase (PKA) also phosphorylates SRSF1, but the effect of this phosphorylation is not well characterized.^{24,27}

The cAMP/PKA signaling pathway is involved in the regulation of numerous cellular processes such as cell growth and differentiation, metabolism, and gene expression. In the absence of cAMP, PKA is an inactive tetramer composed of a regulatory (R) subunit dimer and 2 catalytic (C) subunits. PKA subunits are encoded by a family of R and C subunit genes (RI α , RII α , RI β , RII β , C α , C β , C γ , and PRKX).²⁸ Specificity in this signal transduction pathway is obtained by compartmentalization by A-kinase anchoring proteins (AKAPs) and by expression of multiple forms of

the R and C subunits, which are expressed in a tissue-specific manner and harbor different physiochemical properties.^{29,30} Several C subunit-binding proteins have also been identified during recent years.^{29,31-34} PKA is activated upon binding of 4 molecules of cAMP to the R dimer, releasing the C subunits to phosphorylate relevant substrates on serine and threonine residues in its vicinity.³⁵ However, a proportion of the C subunit translocates to the nucleus after activation, where it is involved in the regulation of transcription and splicing.³⁶⁻³⁸ We have previously shown that PKA C α 1 is involved in the regulation of pre-mRNA splicing *in vivo*, possibly through phosphorylation of splicing factors, because the C subunit was shown to phosphorylate SR proteins *in vitro*.³⁸ We observed that the band representing SR proteins with molecular weight around 30 kDa, such as SRSF1, SRSF2, and SRSF9, had the highest amount of ³²P incorporated.

Here, we have analyzed SRSF1 for potential sites for PKA phosphorylation and identified a site at serine 119. We also demonstrated an interaction between SRSF1 and PKA C α 1 that was not dependent on serine 119 phosphorylation but that required active PKA C α 1. Furthermore, mutation of serine 119 altered SRSF1's ability to bind RNA *in vivo* and enhanced its ability to promote pre-mRNA splicing of the Minx transcript *in vitro*, implying that PKA may inhibit the ability of SRSF1 to activate splicing.

Results

SRSF1 is phosphorylated by PKA *in vitro* and *in vivo*, but the actual phosphorylation site(s) are not identified.^{24,27} We analyzed the SRSF1 sequence (Uniprot:Q07955) for potential PKA phosphorylation sites, and the NetPhosK Server³⁹ identified 4 putative PKA phosphorylation motifs at serines 119, 231, 242, and 246 (scoring between 0.51-0.77) (Fig. 1). It should be noted that serine 119 is the only site located outside of the RS domain. Based on this, we initially investigated which serine residues in SRSF1 become phosphorylated by PKA. Several plasmids of SRSF1 for prokaryotic expression were made. In order to test serine 119 as a potential phosphorylation site, one of the constructs made was devoid of the RS domain (SRSF1 Δ RS) (Fig. 1, lower sequence). The RS domain was removed because we suspected that the high number of arginine/serine repeats would lead to unspecific phosphorylation under our experimental conditions that could conceal phosphorylation outside of the RS domain. We also consider serine 119 as the most likely PKA phosphorylation site for phosphorylation *in vivo* because the serine residues in the RS domain are extensively phosphorylated by the SR protein-specific kinases SRPK1 and Clk/Sty via processive mechanisms.⁴⁰⁻⁴² This residue was therefore mutated to alanine both in the full-length (SRSF1 S119A) and the RS-deleted (SRSF1 Δ RS S119A) constructs. After recombinant expression of

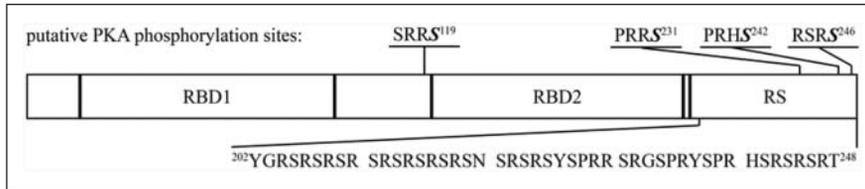


Figure 1. Domains and putative PKA phosphorylation sites in SRSF1. Schematic representation of SRSF1 showing the sequence and location of the 4 putative PKA phosphorylation sites predicted by the NetPHosK server. The deleted sequence in SRSF1 Δ RS (aa 202-248) is expanded.

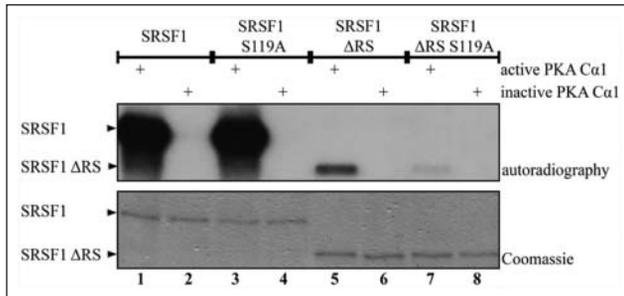


Figure 2. PKA phosphorylates SRSF1 at serine 119 *in vitro*. Purified SRSF1 (lanes 1 and 2), SRSF1 S119A (lanes 3 and 4), SRSF1 Δ RS (lanes 5 and 6), and SRSF1 Δ RS S119A (lanes 7 and 8) were incubated with active or heat-inactivated PKA α 1 and γ -[32 P]-ATP in a reaction buffer. The samples were analyzed by SDS-PAGE followed by Coomassie staining (lower panel) and autoradiography (upper panel).

the 4 SRSF1 constructs, the protein products were subjected to *in vitro* phosphorylation. The full-length SRSF1 wild-type (Fig. 2, lanes 1 and 2) and S119A-mutated (Fig. 2, lanes 3 and 4) proteins were both heavily phosphorylated by PKA as determined by the incorporation of 32 P in the presence of active but not inactive PKA α 1 and γ -[32 P]-ATP. No apparent reduction in the phosphorylation levels due to the mutation at serine 119 was observed. However, when the RS domain was removed (Fig. 2, lanes 5 and 6), the protein was still phosphorylated but to a lower extent than the full-length proteins. This suggested that serine 119 may be a site in SRSF1 for PKA phosphorylation, a suggestion that was supported by markedly reduced phosphorylation when SRSF1 Δ RS S119A was incubated with PKA α 1 in the presence of γ -[32 P]-ATP (Fig. 2, lanes 7 and 8).

PKA α 1 and SRSF1 are both located in splicing factor compartments.^{38,43} To explore whether the S119A mutation altered subcellular localization, we conducted immunofluorescence experiments of U2OS cells transfected with full-length wild-type and S119A-mutated SRSF1. Cells were fixated and permeabilized 16 hours after transfection and stained with anti-SRSF1 and Hoechst. Both SRSF1 wild-type (Fig. 3, upper panel) and SRSF1 S119A (Fig. 3, lower panel) were localized in the nucleus, and we did not observe any apparent changes in subnuclear localization. In a recent paper, it was shown by GST pull-down and co-immunoprecipitation (co-IP) that α 1 targets SRSF1.²⁷

Based on the results depicted in Figure 2, we tested whether PKA phosphorylation of SRSF1 at serine 119 regulates this interaction. We first verified the interaction by cross-IP of co-transfected PKA α 1 and SRSF1 in 293T cells using anti-SRSF1 (Fig. 4A, lane 3) and anti-PKA α (Fig. 4B, lane 3). PKA α 1 was further co-transfected with SRSF1 S119A and subjected to the

same cross-IP using anti-SRSF1 (Fig. 4A, lane 6) and anti-PKA α (Fig. 4A, lane 9). This demonstrated that the S119A mutant of SRSF1 still interacted with active α 1, indicating that serine 119 may not be important for the interaction. To further investigate the role of PKA in these interactions, we introduced an inactive form of α 1 mutated at lysine 73 (lysine 72 when the first methionine is not included) (PKA α 1 K73M).⁴⁴ In these experiments, the interaction was abolished, as anti-PKA α was only able to immunoprecipitate PKA α 1 (Fig. 4B, lower panel, lane 6) and not SRSF1 (Fig. 4B, upper panel, lane 6). This result was confirmed by applying anti-SRSF1, as this antibody was able to immunoprecipitate SRSF1 but not PKA α 1 K73M (Fig. 4B, lane 9). Taken together, this firstly demonstrated that wild-type SRSF1 and PKA α 1 interact in 293T cells. Secondly, mutation of PKA α 1 at lysine 73 abolished not only α 1 activity as previously reported⁴⁴ but also the interaction of α 1 with SRSF1.

This made us want to investigate other roles of serine 119 in SRSF1 function. It has previously been shown that phosphorylation of RNA binding proteins can change their RNA binding properties.⁴⁵⁻⁴⁸ In fact, it was recently demonstrated that PKA phosphorylation of SRSF1 enhances binding to tau pre-mRNA.²⁷ The serine 119 PKA phosphorylation site in SRSF1 is located close to the N-terminal end of RBD2 (Fig. 1). Furthermore, a structural analysis of RNA binding to SRSF1 showed that when the 2 arginines preceding serine 119 are mutated, RNA binding is severely reduced.⁴⁹ Hence, serine 119 may potentially be involved in regulating RNA binding. To test this, we conducted RNA-IP with transfected SRSF1 and SRSF1 S119A. This confirmed that wild-type SRSF1 binds RNA (Fig. 5A, lane 2) whereas mutation of serine 119 reduced RNA binding significantly ($P = 0.02$; $n = 3$) (Fig. 5A [lane 4] and 5B).

Knowing that mutation of serine 119 affects SRSF1's RNA-binding capacity, we next investigated if this mutation also affects SRSF1-dependent pre-mRNA splicing. Due to the high endogenous levels of SRSF1, it is difficult to analyze the effect of the S119A mutant by an *in vivo* minigene splicing assay or *in vitro* splicing assays using nuclear extracts. We therefore took advantage of an *in vitro* S100 complementation assay using adenoviral Minx pre-mRNA as a transcript. This model pre-mRNA derived from

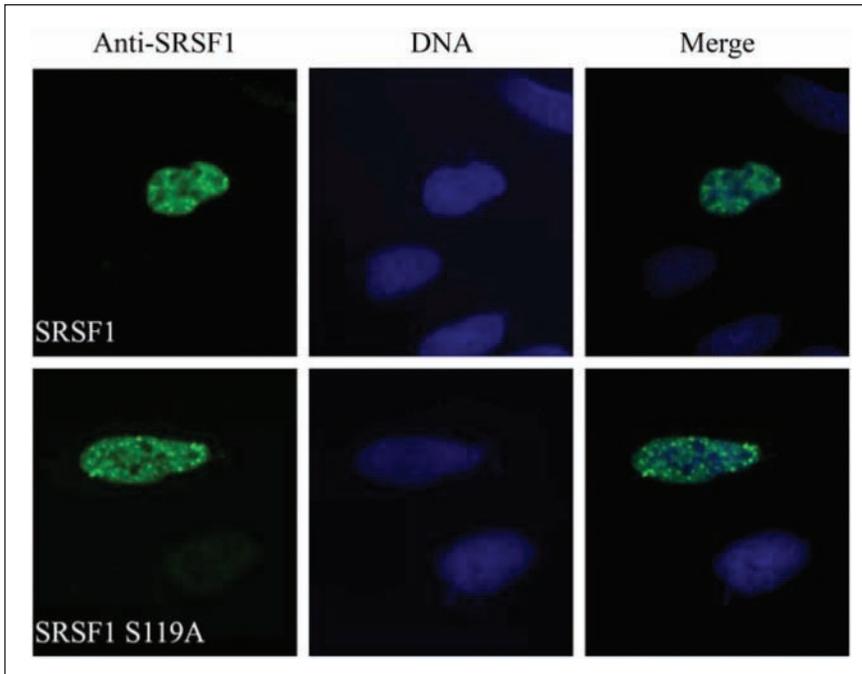


Figure 3. SRSF1 and SRSF1 S119A are localized in the nucleus. Immunofluorescence of PF-fixed U2OS cells transfected with SRSF1 wild-type (upper panel) or SRSF1 S119A (lower panel) and stained with anti-SRSF1 (left column, green). DNA was visualized by Hoechst staining (middle column, blue). Photograph overlays are shown to the right (Merge).

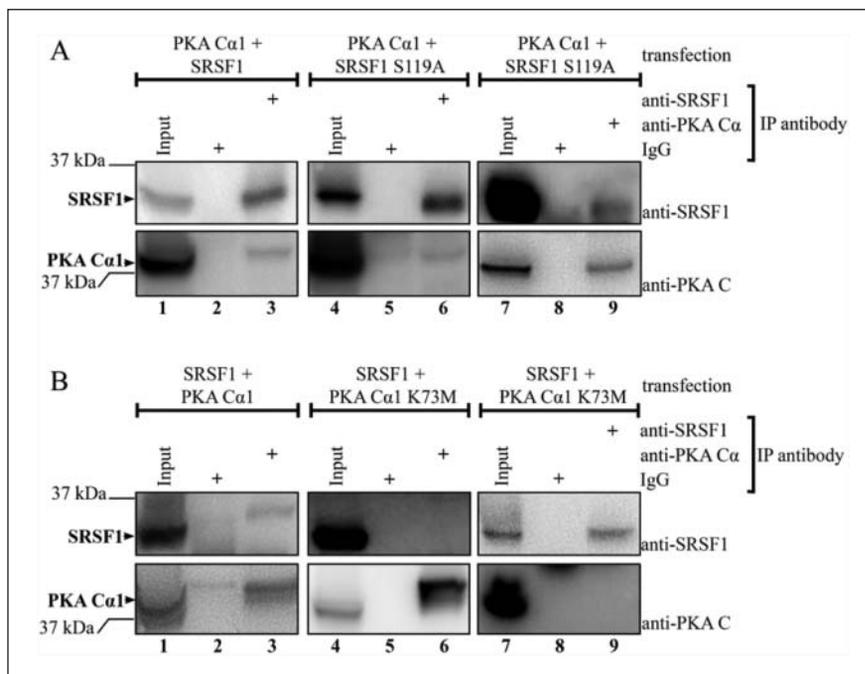


Figure 4. SRSF1 interacts with catalytically active PKA α 1. **(A)** 293T cells were co-transfected with either PKA α 1 and SRSF1 (lanes 1-3) or PKA α 1 and SRSF1 S119A (lanes 4-9). The cell lysates were adjusted to equal protein concentration for each experiment and precleared with magnetic beads before input samples were collected (lanes 1, 4, and 7). The lysates were subjected to IPs using anti-SRSF1 (lanes 3 and 6), anti-PKA α (lane 9), mouse IgG (lanes 2 and 5), or rabbit IgG (lane 8) and magnetic beads. All samples were analyzed by SDS-PAGE and immunoblotting using anti-SRSF1 (upper panel) and anti-PKA C (lower panel). **(B)** 293T cells were co-transfected with either SRSF1 and PKA α 1 (lanes 1-3) or SRSF1 and PKA α 1 K73M (lanes 4-9). The cell lysates were adjusted to equal protein concentration for each experiment and precleared with magnetic beads before input samples were collected (lanes 1, 4, and 7). The lysates were subjected to IPs using anti-PKA α (lanes 3 and 6), anti-SRSF1 (lane 9), mouse IgG (lane 8), or rabbit IgG (lanes 2 and 5) and magnetic beads. All samples were analyzed by SDS-PAGE and immunoblotting using anti-SRSF1 (upper panel) and anti-PKA C (lower panel).

the major late transcription unit of adenovirus is known to be spliced in cytoplasmic S100 extracts complemented with SRSF1.⁵⁰ We therefore considered the Minx pre-mRNA as well suited for the analysis of differences in splicing capacity between SRSF1 and SRSF1 S119A. As controls for these experiments, we performed splicing of the Minx transcript in HeLa nuclear extracts (Fig. 6A, lane 1), uncomplemented S100 extract (Fig. 6A, lane 2), and SR protein-complemented S100 extract (Fig. 6A, lane 3). This showed a minimal amount of splicing activity in uncomplemented compared to the complemented S100 extract (Fig. 6A, lanes 2 and 3). These controls provided a threshold for monitoring the relative contribution of wild-type and mutated SRSF1 to pre-mRNA splicing (Fig. 6A, lanes 4 and 5). The results revealed that the SRSF1 S119A mutant protein had a significantly higher capacity to splice the Minx pre-mRNA in the S100 extract compared to the wild-type protein ($P = 0.04$; $n = 9$) (Fig. 6B).

Discussion

Here, we present evidence that PKA phosphorylates the splicing factor SRSF1 at serine 119 *in vitro*. Prevention of phosphorylation at this site led to a reduction of SRSF1's capacity to bind RNA *in vivo* and increased its capability to splice the Minx transcript *in vitro*. We also present evidence that PKA α 1 and SRSF1 interact by a mechanism that is independent of phosphorylation at serine 119 but that requires active PKA. PKA phosphorylation of full-length SRSF1 is documented in 3 independent reports.^{24,27} The NetPhosK server depicted 4 possible PKA phosphorylation sites, of which the consensus sequence for the first 3 is RXS (arginine-random-serine), while the last one is RXXS (arginine-random-random-serine). These consensus sites have been postulated to have a relatively low probability of being phosphorylated.⁵¹ However,

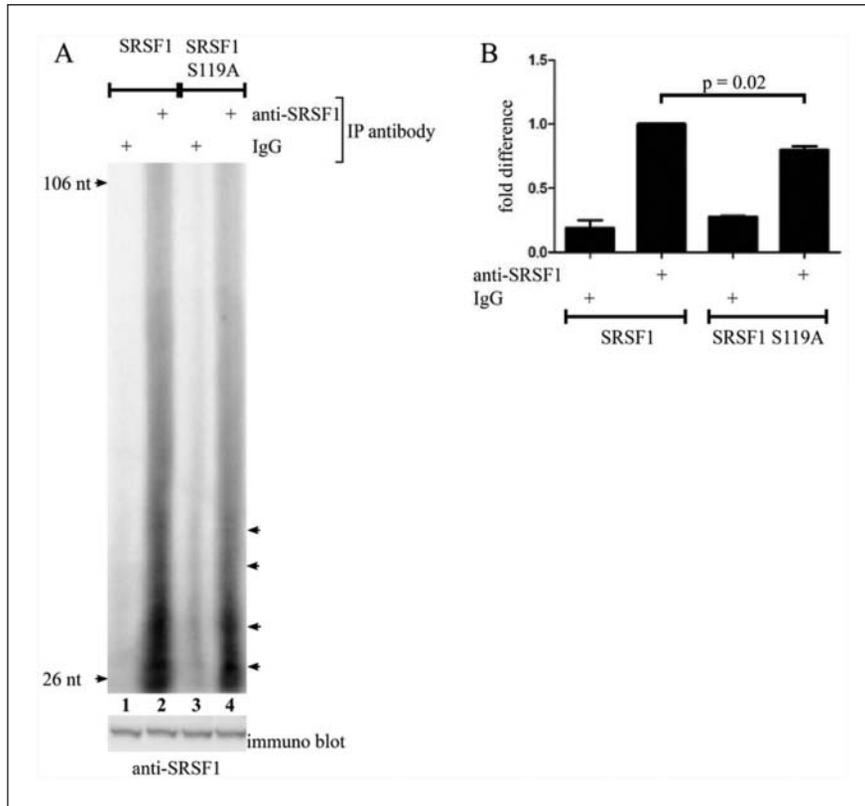


Figure 5. Mutation of serine 119 decreases the ability of SRSF1 to interact with RNA. **(A)** 293T cells were transfected with SRSF1 (lanes 1 and 2) or SRSF1 S119A (lanes 3 and 4). Twenty hours posttransfection, the cells were UV cross-linked and lysed. The cell lysates were adjusted to equal protein concentration and treated with T1 RNase before IPs with magnetic beads conjugated with either mouse IgG (lanes 1 and 3) or anti-SRSF1 (lanes 2 and 4). Immunoprecipitated samples were dephosphorylated, labeled with γ -[32 P]-ATP by PNK kinase, and run on a denaturing polyacrylamide gel before analysis by autoradiography. The immunoblot (lower panel) shows the amount of SRSF1 in the cell lysate. The arrows indicate accumulation of specific RNA species. **(B)** Lanes in unsaturated images were manually detected in Adobe Photoshop using identical frames. The obtained intensities were adjusted for background and analyzed in GraphPad Prism by a paired t test ($n = 3$).

there are several examples of physiological PKA substrates with these consensus sites, such as elongation factor-2 kinase (PRRS⁴⁹⁹),⁵² hormone-sensitive lipase (MRRS⁵⁶³ and PRRS⁶⁵⁹),⁵³⁻⁵⁵ heat shock factor 1 (RPSS³²⁰),³³ and *hSLO* BK_{Ca} α subunit of large conductance Ca²⁺-dependent K⁺ channel (RQPS⁸⁶⁹).⁵⁶ Based on this, we cannot rule out that SRSF1 is phosphorylated at any of these sites in the RS domain, especially because S119A-mutated full-length SRSF1 was strongly phosphorylated by active PKA α 1 *in vitro*. Furthermore, Shi *et al.* also showed that the RS domain is extensively phosphorylated by PKA *in vitro*, and they observed reduced phosphorylation with increasing deletions of the RS domain.²⁷ It is, however, difficult to determine the importance of phosphorylation of the RS domain due to the high probability of unspecific phosphorylation under *in vitro* conditions, where PKA may act in a promiscuous way. To address this further, one would have to mutate every putative PKA phosphorylation site

individually and accordingly analyze alterations in SRSF1 function *in vivo* systematically.

That PKA α 1 and SRSF1 interact confirms results from a recent paper by Shi *et al.* where they show that GST-tagged SRSF1 pulls down PKA α 1 and that anti-HA immunoprecipitated PKA α 1 together with several HA-tagged SRSF1 variants.²⁷ In the present study, we immunoprecipitated untagged PKA α 1 with anti-SRSF1 and *vice versa* and were therefore able to verify the interaction by cross-IPs. In these experiments, we also observed that mutation of serine 119 to alanine, which prevents phosphorylation, did not abrogate the interaction. This indicates that this phosphorylation event is not essential for interaction. The amount of co-immunoprecipitated proteins was relatively low, however, not unexpected, taking into account the relatively low abundance of PKA C subunits in the nucleus compared to the whole cell³⁸; hence, only a small fraction of the C subunit will be associated with SRSF1 at all times. When transfected into 293T cells, the C subunit is expressed at high levels in the cytoplasm (results not shown). Shi *et al.* documented that an SRSF1 deletion mutant lacking the most C-terminal end of the RS domain (aa 220-248)

did not show any reduction in the amount of pulled-down PKA α 1 compared to the full-length protein. They also showed that the RS domain alone was not able to immunoprecipitate detectable levels of PKA α 1. All together, this implies that phosphorylation of neither serine 119 nor any of the putative PKA sites in the RS domain is required for the interaction between SRSF1 and PKA α 1. More detailed mapping studies will be required to fully explain the basis for the interaction between the 2 proteins. Interestingly, we show that the interaction actually requires that PKA α 1 is catalytically active because mutation of lysine 73 to methionine abrogated the interaction. Lysine 73 is conserved throughout the whole family of eukaryotic protein kinases, and replacement of this lysine, which is involved in ATP binding, has become a standard approach for establishing the importance of kinase activity.⁵⁷ Lysine 73 is located in β strand 3 in the small lobe, and after mutation of this site, the protein is less stable than the wild-type,

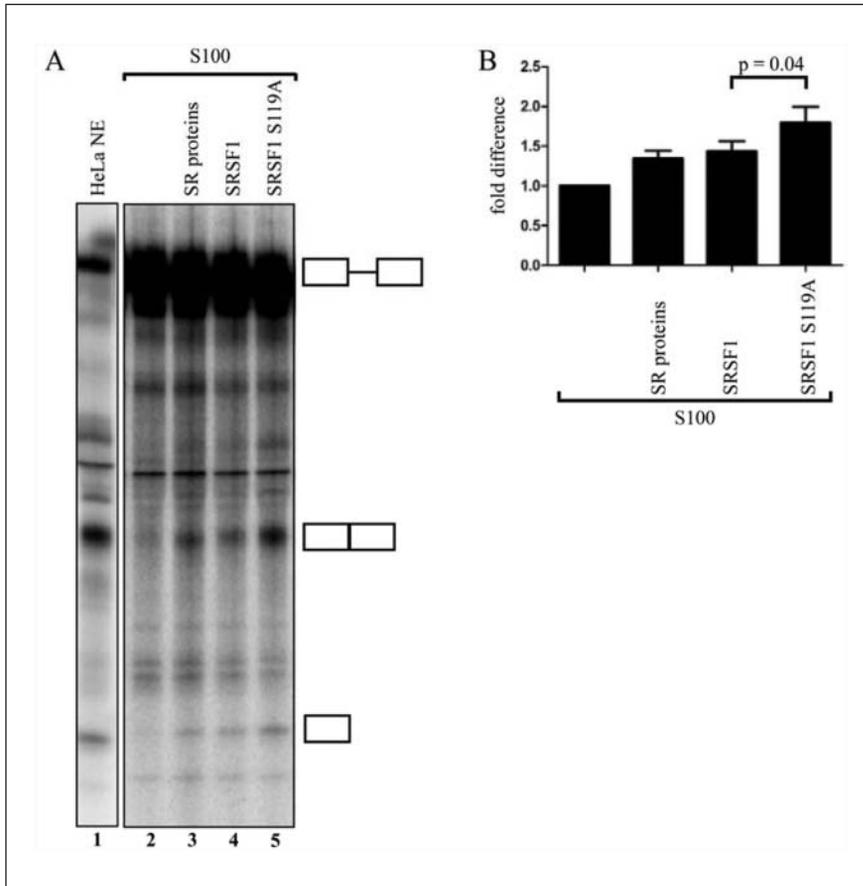


Figure 6. Mutation of serine 119 enhances the ability of SRSF1 to activate splicing of the Minx transcript *in vitro*. **(A)** *In vitro* splicing of Minx pre-mRNA in HeLa-NE (lane 1) or S100 extracts (lanes 2-5). The splicing-deficient S100 extracts were supplemented with either no addition (lane 2), SR mix (lane 3), SRSF1 (lane 4), or SRSF1 S119A (lane 5). The identities of the RNAs are shown schematically at the right side of the panel. **(B)** Unsaturated images were quantified in Scion Image, adjusted for background, and analyzed by a paired *t* test in GraphPad Prism ($n = 9$).

lacks catalytic activity, but is still able to bind ATP.⁵⁸ The unphosphorylated lysine 73 mutant is unable to bind RI and RII subunits, but when it is activated by phosphorylation at threonine 197 in the activation loop, these interactions are restored. The PKA $\text{Ca}1$ K73M mutant is, however, not able to finish the activation cascade that is initiated by threonine 197 phosphorylation.⁵⁹ The interaction between PKA $\text{Ca}1$ and SRSF1 is abolished along with mutation of lysine 73. This may implicate that binding of SRSF1 requires the conformational changes that an active PKA $\text{Ca}1$ displays, such as a stable small lobe with high affinity-bound ATP and completed activation cascade. SRSF1 may interact with PKA $\text{Ca}1$ through the hydrophobic pocket, as is the case for RI, RII, and PKI.⁶⁰ When the crystal structure of SRSF1 is solved, this interaction will be more accessible for investigation.

Based on the localization of serine 119 in proximity to RBD2, we assumed that phosphorylation of this site could be involved in the regulation of RNA binding. Both RBDs

in SRSF1 are capable of binding RNA in the absence of other factors.¹⁸ Our RNA-IP results show that when serine 119 is mutated to alanine, the total amount of RNA bound to SRSF1 is reduced. The actual effect is probably higher because there are endogenous levels of SRSF1 that could mask the effect of the mutation. The measured reduction in bound RNA due to lack of phosphorylation was approximately 20%. This may imply that this phosphorylation event does not lead to a switch in activity but rather acts as a modulator of RNA binding. Interestingly, it has been shown that SRSF1 is bridged to other RNA binding proteins, such as U1-70K, through binding via the RBDs.⁶¹ Whether the RNA IPs presented here are influenced by or that S119 phosphorylation is involved in such a mechanism will need further investigation. The results documented here indirectly suggest that PKA phosphorylation at serine 119 enables SRSF1 to bind broader spectra of RNA. In fact, this correlates well with the report showing that PKA phosphorylation enhances the binding of SRSF1 to exon 10 of tau pre-mRNA.²⁷ This result is, however, only valid if S119 really is phosphorylated by PKA in the intact cell.

For constitutive splicing regulated by SRSF1, both RBDs and the RS domain are required. In the regulation of alternative splicing, and more specifically for 5' splice-site selection, RBD1 and RBD2 are sufficient.¹⁸ The *in vitro* splicing assay with the Minx transcript demonstrated an increased amount of splice product when serine 119 was mutated to alanine, indicating that PKA phosphorylation of this particular site inhibits the ability of SRSF1 to activate splicing. We cannot, however, rule out that lack of phosphorylation by other kinases influences the effect we observe; however, this will need further investigation. To our knowledge, there are no reports describing a direct interaction between PKA $\text{Ca}1$ and RNA or that PKA $\text{Ca}1$ (Uniprot P17612) contains any RBDs. We, therefore, assume that PKA $\text{Ca}1$'s involvement in pre-mRNA splicing occurs indirectly by interaction and phosphorylation of other proteins, such as SRSF1. We have previously described that PKA $\text{Ca}1$ promotes distal 5' splice-site selection of the E1A minigene *in vivo*³⁸ and that splicing factor

μL . The reactions were incubated at 30°C for 30 minutes on slushy ice for 1 hour and stopped by adding SDS loading dye and boiled for 5 minutes. The proteins were resolved by SDS-PAGE and analyzed by Coomassie staining and autoradiography.

Cell culture. 293T cells were maintained at 37°C in humidified air with 5% CO₂ in RPMI 1640 (cat. no. R0883, Sigma-Aldrich, Oslo, Norway) supplemented with 10% fetal bovine serum (cat. no. F7524, Sigma-Aldrich), 1% nonessential amino acids (cat. no. 11140-035, Invitrogen Dynal AS), 1% L-glutamine (cat. no. G7513, Sigma-Aldrich), 1% sodium pyruvate (cat. no. 11360-039, Invitrogen Dynal AS), and 1x penicillin-streptomycin solution (cat. no. P4458, Sigma-Aldrich). U2OS cells were grown in DMEM (cat. no. D6546, Sigma-Aldrich) supplemented with 10% fetal bovine serum (cat. no. F7524, Sigma-Aldrich), 1% L-glutamine (cat. no. G7513, Sigma-Aldrich), and 1x penicillin-streptomycin solution (cat. no. P4458, Sigma-Aldrich). The cells were transfected with Fugene HD (cat. no. 04 709 705 001, Roche Diagnostics, Oslo, Norway) according to the manufacturer's protocol. Briefly, 6 μL Fugene HD (cat. no. 04 709 705 001, Roche Diagnostics), 2 μg plasmid DNA, and 100 μL Opti-MEM (cat. no. 31985, Invitrogen Dynal AS) were mixed and added to each well (10 cm²) and incubated for 20 hours.

Immunofluorescence. Transfected U2OS cells grown on cover slips were washed with phosphate-buffered saline (PBS) and fixed/permeabilized with 3% paraformaldehyde and 0.1% Triton X-100 in PBS. After being washed with PBS containing 0.1% Tween 20 (PBST), the cover slips were blocked with PBST containing 2% bovine serum albumin (PBST-BSA) for 15 minutes. Anti-SF2/ASF (mab 96, cat. no. 32-4500, Invitrogen Dynal AS) was diluted 1:50 in PBST-BSA and incubated for 30 minutes. The cover slips were washed extensively before incubation for 30 minutes and Alexa Fluor 594 goat anti-mouse IgG (1:400, cat. no. A11005, Invitrogen Dynal AS) secondary antibody. Finally, the cover slips were washed with PBST-BSA, and 0.5 g/mL Hoechst (cat. no. B2261, Sigma-Aldrich) was added in the last wash for DNA staining. The cover slips were mounted in Citifluor AF1 (Citifluor Ltd., London, United Kingdom) and examined using an Olympus BX61 microscope attached with an F-VIEW digital camera (Olympus, Tokyo, Japan). Photographs were acquired on the computer using analysis (Soft Imaging Systems, Münster, Germany).

Co-immunoprecipitation (co-IP). Four wells with co-transfected 293T cells were washed in PBS and lysed by sonication in IP buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 0.5% Triton X-100, 1x protease inhibitor cocktail [cat. no. P8340, Sigma-Aldrich], 1 mM PMSF, and 1 mM Na₃VO₄). The lysates were cleared by centrifugation and adjusted to

equal protein concentration for each experiment (1.7-2.9 μg protein/ μL) by the Bradford method (BioRad, Oslo, Norway). The lysates were precleared with Dynabeads Protein G (1:10, cat. no. 100-04D, Invitrogen Dynal AS) before input samples were collected (20 μL), and the remainder of the lysates were incubated with one of the following antibodies (2.5 μg /sample): mouse IgG (cat. no. I5381, Sigma-Aldrich), rabbit IgG (cat. no. I5006, Sigma-Aldrich), anti-SF2/ASF (cat. no. 32-4500, Invitrogen Dynal AS), or anti-PKA α cat (cat. no. SC-903, Santa Cruz Biotechnology, Santa Cruz, CA). The mixture of cell lysate and antibody was left incubated at 4°C rotating overnight before Dynabeads Protein G (1:10, cat. no. 100-04D, Invitrogen Dynal AS) was added the next day and incubated for another hour. The samples were washed with IP buffer, added with SDS loading dye, and boiled for 5 minutes before immunoblotting.

Immunoblotting. Samples were separated by SDS-PAGE and transferred to PVDF membrane by electroblotting. The membrane was blocked by drying and rehydration in methanol before incubation with primary antibodies diluted in TBST (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20), anti-SF2/ASF (30 μg , cat. no. 32-4600, Invitrogen Dynal AS), or anti-PKA_C (1:250, cat. no. 610981, BD Biosciences Transduction Laboratories, Erembodegem, Belgium). The membrane was washed in TBST before incubation with the secondary antibodies diluted in TBST, HRP-conjugated goat anti-mouse (1:2,000, cat. no. 55563, MP Biomedicals, Irvine, CA), or HRP-conjugated goat anti-rabbit (1:2,000, cat. no. 55689, MP Biomedicals). After washing with TBST, immunoreactive proteins were visualized by ECL (cat. no. 34076, Pierce, Rockford, IL) and developed using the G:BOX imaging system (Syngene, Cambridge, United Kingdom).

RNA IP. The method is according to Rogne *et al.*^{45,70} In brief, one sample is the extract of four 10-cm² wells of transfected 293T cells. Culture medium was replaced by PBS before the cells were UV irradiated (254 nm, 200 mJ/cm², twice), harvested, washed in PBS, and lysed by sonication in 300 μL IPB buffer (10 mM HEPES, pH 7.5, 2 mM EDTA, 10 mM KCl, 1% TX-100, 1 mM PMSF, and 1x protease inhibitor cocktail [cat. no. P8340, Sigma-Aldrich]) supplemented with 150 mM NaCl. Lysates were cleared by centrifugation before addition of T1 RNase (final concentration of 0.025 U/ μL , cat. no. EN0541, Fermentas, St. Leon-Rot, Germany) and incubation for 6 minutes at 37°C with 1,000-rpm agitation. The lysates were adjusted to equal protein concentrations by the Bradford method and precleared with Dynabeads Protein G (1:10, cat. no. 100.04D, Invitrogen Dynal AS) for 30 minutes at 4°C rotating. Samples for immunoblotting were collected, added with SDS loading dye, and boiled for 5 minutes. Dynabeads

Protein G (1:10, cat. no. 100.04D, Invitrogen Dynal AS) was conjugated with anti-SF2/ASF (2.5 µg, cat. no. 32-4600, Invitrogen Dynal AS) or mouse IgG (2.5 µg, cat. no. I5381, Sigma-Aldrich) for 2 hours at 4°C followed by 3 washes in IPB buffer. Supernatants were incubated with 30 µL conjugated beads for 2 hours at 4°C rotating. The immunoprecipitated samples were washed 4 times by alternating between 1 mL RIPA (1x PBS, 0.05% SDS, 0.1% deoxycholate, 1% NP-40, 1x protease inhibitor cocktail [cat. no. P8340, Sigma-Aldrich]) and RIPA 1000 (as RIPA buffer but supplied with 1 mM EDTA and 1 M NaCl). The immunoprecipitated samples were dephosphorylated by calf intestinal phosphatase (final concentration of 0.038 U/µL, cat. no. 10713023001, Roche Diagnostics) for 10 minutes and washed 3 times with Buffer C (50 mM Tris HCl, pH 7.4, 10 mM MgCl₂, 0.5% NP-40, 1x protease inhibitor cocktail [cat. no. P8340, Sigma-Aldrich]), followed by labeling with γ-[³²P]-ATP (2.1 µCi, cat. no. NEG502H, Perkin Elmer, Maanstraat, Germany) by polynucleotide kinase phosphorylation (0.5 U/µL, cat. no. M0201L, New England Biolabs Inc., Ipswich, MA) and further washed 3 times with Buffer C. Samples were resolved on 6% denaturing polyacrylamide gels. Incorporated phosphate was visualized by autoradiography. Unsaturated images from the Typhoon 9410 phosphoimager (GE Healthcare Life Sciences) were quantified using the histogram function in Adobe Photoshop (San Jose, CA). The lanes were manually defined with identical frames. The statistical analysis was performed with a paired Student *t* test in Prism GraphPad (La Jolla, CA).

Transcript synthesis and in vitro splicing. Radiolabeled Minx pre-mRNA was generated by T7 RNA polymerase transcription of a BamHI-cut PCR-amplified product from pMINX MS2⁷¹ as previously described.⁷² Nuclear and S100 extracts were prepared from HeLa spinner cells as previously described.⁷² The total splicing reaction volume was 25 µL and contained approximately 20 to 25 fmol ³²P-labeled Minx transcript, 8 µL nuclear or S100 extracts, 2.6% polyvinyl alcohol, 12% glycerol, 12 mM HEPES-KOH (pH 7.9), 60 mM KCl, 2 mM ATP, 20 mM creatine phosphate, 2.5 mM MgCl₂, and 0.3 mM DTT. Reactions were incubated at 30°C for 2 hours, treated with proteinase K, and phenol extracted. After sodium acetate/ethanol precipitation, the RNA was resolved on 6% denaturing polyacrylamide gels, followed by autoradiography. Unsaturated images from the Typhoon 9410 phosphoimager (GE Healthcare Life Sciences) were quantified using Scion Image (Scion Corporation) and adjusted for background. The statistical analysis was performed with a paired Student *t* test in Prism GraphPad.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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