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Phosphorylation by SR kinases regulates the binding of PTB-associated splicing factor (PSF) to the pre-mRNA polypyrimidine tract

Ching-Jung Huang¹, Zhaohua Tang^{2,3}, Ren-Jang Lin², and Philip W. Tucker^{1,*}

¹Section of Molecular Genetics and Microbiology and Institute for Cellular and Molecular Biology, University of Texas, Austin, TX 78712

²Division of Molecular Biology, Beckman Research Institute of the City of Hope, Duarte, CA 91010

Abstract

PSF (PTB-associated splicing factor) is a multi-functional protein that participates in transcription and RNA processing. While phosphorylation of PSF has been shown to be important for some functions, the sites and the kinases involved are not well understood. Although PSF does not contain a typical RS domain, we report here that PSF is phosphorylated *in vivo* to generate an epitope(s) that can be recognized by a monoclonal antibody specific for phosphorylated RS motifs within SR proteins. PSF can be phosphorylated by human and yeast SR kinases *in vivo* and *in vitro* at two isolated RS motifs within its N terminus. A functional consequence of SR phosphorylation of PSF is to inhibit its binding to the 3' polypyrimidine tract of pre-mRNA. These results indicate that PSF is a substrate of SR kinases whose phosphorylation regulates its RNA binding capacity and ultimate biological function.

INTRODUCTION

The removal of intervening sequences (introns) from pre-mRNA (splicing) is catalyzed by the spliceosome. The formation of the spliceosome happens in a stepwise manner via the organization of small ribonucleoproteins (snRNPs), U1, U2, U4/U6 and U5 and non-snRNP proteins. Following U1 snRNP association with the 5' splice site, SF1 and U2AF⁶⁵ cooperatively bind to the branch point and polypyrimidine tract (Py) to recruit U2snRNP to form the A complex. The incorporation of tri-snRNPs, U4/U6.U5 is followed by spliceosome rearrangement to form the catalytic center, C complex [1,2]. In higher eukaryotes, the Py of the 3' splice site is highly conserved and recognized by several proteins, including U2AF⁶⁵ and PSF [1,3]. U2AF⁶⁵, a member of the SR family[4], through recognition of the branch point and Py, can recruit U2 snRNA to base-pair with the branch sequence [3]. PSF is a 100 kDa protein, which was originally co-purified with Py binding protein (PTB) [5]. Subsequently, Patton et al. [6] found that PSF, but not PTB, could interact with the Py independently.

Several lines of evidence have shown that PSF is a multi-functional protein [7]. PSF is generally isolated in a complex [8] with a similar, multifunctional RNP protein, p54^{nrB}/nonO [9,10]. Both contain the DBHS (Drosophila Behavior; Human Splicing) domain, a region present in

*To whom correspondence should be addressed: Molecular Genetics and Microbiology, University of Texas at Austin, 1 University Station A5000 Austin, Texas 78712. Phone: 512.475.7705; Fax: 512.475.7707; Email: philtucker@mail.utexas.edu

³Current address: W. M. Keck Science Center, The Claremont College, Claremont, CA 91711

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several other proteins that function in quite different ways to regulate gene expression [7,9, 11]. In addition to its Py binding, PSF as a complex with p54^{nrb}/nonO, binds to U5snRNA, indicating a role in spliceosome formation [12]. Its association with U4/U6.U5 tri-snRNP [12,13] further indicates that PSF participates in the second catalytic step of splicing [14]. Transcription and pre-mRNA splicing are coupled by the C-terminal domain (CTD) of RNA polymerase II [15]. PSF and p54^{nrb}/nonO bind strongly to both the hypo- or hyper-phosphorylated forms of the CTD *in vivo* and *in vitro* [16]. Strong transcriptional activators enhance levels of splicing and 3'-end cleavage, and this stimulation requires the CTD [17]. PSF but not p54^{nrb}/nonO preferentially binds to strong activation domains to mediate transcriptional activator- and CTD-dependent stimulation of pre-mRNA processing [18]. As further evidence for a function in transcriptional initiation, PSF forms a ternary complex with the DNA-binding domains of several nuclear hormone receptors and with Sin3A to recruit class I histone deacetylases (HDACs) for repression [19]. Similarly, PSF and p54^{nrb}/nonO interact with the steroidogenic factor (SF-1) to recruit Sin3A and HDACs to a repressive complex on the human CYP17 promoter [20]. In addition to these effects on splicing and transcription, the PSF-p54^{nrb}/nonO complex appears to function as a double-stranded break rejoining factor [21]. PSF and p54^{nrb}/nonO have been identified as components of a nuclear RNA retention complex for regulating late expression of polyoma RNAs [22]. Finally, PSF binding to a cis-acting regulatory element (INS) within Human Immunodeficiency Virus type I (HIV-1) *gag/pol* and *env* mRNAs leads to the degradation of INS-containing mRNA, whereas binding of p54^{nrb}/nonO to the INS leads to nuclear export of INS-mRNAs into the cytoplasm [23].

The serine/arginine-rich proteins (SR proteins) are a highly conserved family and play roles in both constitutive and alternative pre-mRNA splicing [4]. Typically SR proteins contain at least one RNA recognition motif (RRM) and an RS-rich domain at the carboxyl-terminus. SR proteins can be extensively phosphorylated by kinases of the SRPK and Clk families [24] as well as by topoisomerase I [25]. RS phosphorylation occurs exclusively on serines within the RS motif. 'Non-typical' RS substrates which lack a defined RS domain have not been reported in mammalian cells. However, in yeast, the RGG-containing, RS tract-devoid RNA binding protein, Npl3, has been confirmed as a substrate for the SR kinase, Sky1 *in vivo* and *in vitro* [26,27]. Not only do SR kinases have high specificity of their substrate proteins, but they also show preference toward certain RS context. Wang et al. [28] showed that SRPK2 has a stringent preference for RS dipeptides, and the consensus may be used to predict potential phosphorylation sites in candidate RS domain-containing proteins.

The degree of SR phosphorylation can regulate subcellular localization, protein-protein interactions and the splicing reaction itself [29]. SR proteins are well-known to coordinate protein-protein interactions by their RS domain and recruit other components of splicing [30]. For example, SR proteins interact with exonic splicing enhancer (ESE) sequences [29] and with the intronic branchpoint [31]. SR domains are required for mature spliceosome assembly [31,32], for recruiting the U4/U6.U5 tri-snRNP into the spliceosome [33,34], and for the second catalytic step of splicing [35]. In general, SR proteins have been shown to participate throughout the splicing reaction mediating both protein-RNA and direct RNA contacts throughout the splicing process. Simultaneous collaboration of multiple SR proteins may be required to remove a single intron [35,36].

Both PSF and p54^{nrb}/nonO are phospho-proteins [37-39,40]. Tyrosine-phosphorylated PSF and p54^{nrb}/nonO have been purified from the nuclear envelope of neuroblastoma cells, but the functional consequences were not investigated [37]. PSF and p54^{nrb} can be phosphorylated *in vitro* by protein kinase C (PKC) [38,39]. While this appeared to enhance PSF's non-specific binding to DNA *in vitro*, no *in vivo* confirmation was provided [38]. Hyperphosphorylation of

N-terminal serine/threonine residues in PSF was reported to alter its protein-protein interactions, but neither the kinase nor mechanism was identified [40].

Neither PSF nor p54^{nrb}/nonO contains prototypic RS tracts, but both each contains scattered RS dipeptides. The participation of these two proteins in RNA processing and their multi-functional similarities to SR proteins prompted us to address this issue experimentally. Here we show that PSF can be phosphorylated *in vivo* to induce the formation of an epitope that can be recognized by an anti-SR antibody. PSF, but not p54^{nrb}/nonO, can be phosphorylated *in vitro* by SR kinases, SRPK1 and DSK1 at an RS site within the N-terminus. SR phosphorylation of PSF inhibits its binding to the polypyrimidine tract of pre-mRNA introns. These results indicate that PSF is an unconventional substrate of SR kinases whose phosphorylation regulates its binding activity and its ultimate function in pre-mRNA splicing.

MATERIALS AND METHODS

Plasmid constructions

The cloning of full-length pET15b-PSF is described elsewhere [6]. PSF truncations (1-18, 28-47, 253-272, 331-350 and 400-419) were cloned into pGEX (Amersham) separately, and their inserts were amplified by the polymerase chain reaction (PCR) using pfu DNA polymerase (Stratagene). PCR fragments were cloned into BamHI and EcoRI sites of the vector separately. The ORF of ASF/SF2 was amplified by PCR using pfu DNA polymerase (Stratagene), and the amplified ASF/SF2 PCR fragment was subcloned into the BamHI and XhoI sites of pTrcHis (Invitrogen). pTrcHis-nonO was cloned by the same strategy. To clone pET29a-Dsk1, Dsk1 was amplified by PCR. The PCR product was digested with BamHI and XhoI and then subcloned into pET29a (Novagen). pRSETb-SRPK1 was a generous gift of Xiang-Dong Fu (University Of California, San Diego). p54^{nrb} was obtained from Dr. Krainer (Cold Spring Harbor Laboratories). Glutathione-S-transferase (GST) plasmid is a commercial product (Amersham). To clone pCR3.1-PSF and pCR3.1-PSF (HA), the ORF of PSF was cloned into pCR3.1 (Invitrogen). Then, an HA tag was created at the 3' end of the ORF of PSF.

In vitro phosphorylation assay

Protein substrates were purified and separately incubated at 1 μ M with purified SRPK1, 0.5 μ M, or Dsk1, 0.5 μ M, in a total volume of 20 μ l containing a kinase buffer (50mM Tris-HCl buffer, pH7.4, 10mM MgCl₂, 1mM dithiothreitol) in the presence of 50 μ M ATP and 2 μ Ci of [γ -³²P]ATP at 23°C for 30 min. The reaction was terminated by boiling in SDS-PAGE sample loading buffer. Samples were fractionated on an 8% or a 12.5% SDS-polyacrylamide gel, and protein phosphorylation was detected by autoradiography [41].

Purification of recombinant proteins

SRPK1, Dsk1, and putative substrates (nonO, p54^{nrb}, PSF, and ASF/SF2) were each expressed as N-terminally, 3XHis-tagged proteins. Bacterial strains of interest were grown to an OD₆₀₀ of 0.5-0.7 at 37°C in LB containing ampicillin, kanamycin or both. Cultures were induced with IPTG. The harvested cells were washed with PBS and resuspended in HKI buffer [42] and sonicated. The lysate was centrifuged at 5000 rpm for 25min in an HS4 rotor (Sovall) to separate supernatant and cell debris. The supernatant was incubated with Ni-NTA beads (Qiagen) for 1.5hr at 4°C. The beads were spun down and washed 3 times with HKI buffer. The proteins were eluted with HKI buffer containing 250 mM imidazole. Purified proteins were dialyzed against buffer D (20 mM HEPES-KCl [pH7.9], 100 mM KCl, 0.2 mM EDTA, 20% glycerol (v/v), protease inhibitors) before being aliquoted and frozen at -70°C. Strains expressing GST-fusion proteins were cultured and induced in the same manner, but purified on glutathione-agarose beads in HKE buffer [42] with 5mM of reduced glutathione.

Bacterial co-transformation was performed as described elsewhere [43]. Briefly, proteins of interest were constructed with 6xHis-tagged vectors (ampicillin resistant), where as the kinase, pET29a-Dsk1 (kanamycin-resistant) or pET29a-SRPK1 lacked the 6xHis-tag. Following drug selection and minidigest-confirmation of co-transformed single colonies, the His-tagged proteins were purified on Ni-NTA beads as described earlier.

Expression of PSF in insect cells

To express PSF in insect sf21 cells, a Sall-XhoI fragment, containing the full-length ORF of PSF was ligated into the Sall-XhoI sites of the transfer vector, pFASTBACHTc (Gibco, BRL). Dsk1 was cloned into the transfer vector, pFASTBAC, using the same strategy. Recombinant viral DNA was generated with the BAC-To-BAC HT Expression System (Gibco, BRL) according to the manufacturer's instructions. Recombinant viral DNAs were transfected into sf21 cells by using Cellfectin (Gibco, BRL). Supernatants of transfected cultured cells were collected as recombinant virus stocks. Large-scale infections were performed by infecting 100 ml of sf21 cells ($\sim 10^6$ /ml) in flasks with the virus stock of either PSF alone or PSF with Dsk1. The cells were harvested after 48-72 hrs of infection. PSF protein was purified by virtue of its His-tag protein as described above.

Antibodies

For anti-PSF antisera, full-length PSF with 6xHis was purified from *E. coli* and used for immunizing a rabbit. The rabbit blood was collected six weeks following initial injection and two boosts. Derivation of the SR phosphorylation-specific monoclonal antibody, mAb3C5 was described elsewhere [41,44,45] and was generously provided as ascites fluid by Dr. Turner (University of Birmingham, Birmingham, U.K.). Anti-HA monoclonal antibody (clone 12CA5) was purchased from Roche Molecular Biochemicals. The strain of mAb104 [46] was obtained from ATCC. The supernatant of mAb104 cell culture was used without dilution.

Transient transfection and immunoprecipitation

COS-7 cells were transfected by lipofectin using FuGene6 (Roche Molecular Biochemicals) according to manufacturer's recommendations. 100 μ l of serum-free DMEM (GibcoBRL) was added into 3 μ l of FuGene6 transfectin reagent drop-by-drop and incubated at room temperature for 5 min. The diluted FuGene6 transfectin reagent was then added to 1 μ g of cDNA drop-by-drop and incubated at room temperature for 15 min. The resultant FuGene-cDNA mixture was added to one 100 mm plate of COS-7 cells with 2 ml of fresh medium. At 48 h post-transfection, the transfected cells of each well were harvested and lysed with 150 μ l of LOIPB buffer (25 mM Tris-HCl [pH7.8], 150 mM NaCl, 2 mM EDTA, 0.5% NP40 (v/v)). The lysates were spun and the supernatants were pre-cleared with protein A beads. 400 μ l of the cell extracts was incubated with 4 μ l of anti-HA monoclonal antibody for an hour at 4°C. 30 μ l of protein A beads were added and incubated at 4°C for 30min. The bead complex was collected, washed 3 times with LOIPB buffer, boiled in SDS sample buffer, and then loaded onto a SDS polyacrylamide gel. Protein samples were transferred to a PVDF membrane, and western blots were performed using mab3C5 and anti-PSF polyclonal antisera. Enhanced chemiluminescence (ECL) Western blotting (Amersham Corp.) was performed to detect the protein samples as the manufacturers' instructions.

RNA substrate preparation and in vitro RNA-protein gel shift

All RNA oligos were made by Oligo Therapeutics Inc. Synthesized RNA oligos, b/p+Py (NT4) (5'-UGCUGACCCUGUCCCUUUUUUUUCCACAGCUCG), which contains branch sequence (b/p) (UGC UGAC) and polypyrimidine tract (Py) (CCCUUUUUUUU), and scrambled branch sequence (NT3) (AUGCCCAUCGACCUCAAUAAU), were kinased with [γ - 32 P]ATP for 45min. The samples were mixed with RNA loading buffer (98% formamide,

10 mM EDTA, 0.1% xylene cyanol (v/v), 0.1% bromophenol blue (v/v)), and loaded onto a urea-6% polyacrylamide gel. Purified labeled oligos were eluted and ethanol precipitated. Different concentrations of protein (0.5 to 2 μ M) were incubated with radio-labeled RNA oligos (\sim 1 ng) in binding buffer (20 mM HEPES-KCl [pH7.9], 100 mM KCl, 1 mM DTT, 1u/ μ l ribonuclease (RNase) inhibitor (Life Technologies), 0.2 mM EDTA, 0.5mg/ml tRNA and protease inhibitors (Roche Molecular Biochemicals) for 25min at 30°C. Radiolabeled RNA-protein complexes were analyzed on 0.5X TBE 6% native polyacrylamide gels [47].

RESULTS

PSF expressed in mammalian cells was recognized by phospho-RS specific antibody

To test whether PSF is phosphorylated by SR kinases, we first expressed the protein in COS-7 monkey kidney cells and asked whether the expressed protein could be recognized by phospho-RS specific antibodies such as mAb3C5 [41,44]. The full-length cDNA of human PSF was cloned into a mammalian cell expression vector, pCR3.1, which utilizes a CMV promoter for ectopic over-expression. An HA tag was inserted at the C terminus of the PSF ORF (pCR3.1-PSF-HA) for immunoprecipitation purposes. Either pCR3.1, pCR3.1-PSF or pCR3.1-PSF-HA was transiently transfected into Cos-7 cells. After 48 hr, whole cell lysates were prepared and subjected to anti-HA immunoprecipitation followed by western blotting. In the lysates of pCR3.1-PSF-HA -transfected cells, PSF-HA is recognized by both anti-PSF (Fig 1A, lane 3) and mAb3C5 (Fig 1B, lane 2), whereas no protein is recognized by either antibody in the lysates of mock-transfected cells (Fig 1A, lane 2; Fig 1B, lane 1). To confirm that the immunoprecipitation was specific for the HA-tag, we included the lysates of untagged pCR3.1-PSF-transfected cells in the western blotting against anti-PSF. No band was detected in this sample (Fig 1A, lane 1). These results indicate that an epitope is created on PSF expressed in mammalian cells that is recognized by a monoclonal antibody that is specific for phosphorylated RS motifs.

SR Kinases are sufficient to phosphorylate PSF in *E. coli*

Since PSF was recognized by phospho-RS specific antibody in mammalian cells, we decided to test in bacteria whether a prototypic SR protein kinase is necessary and sufficient (in the absence of other eukaryotic-specific co-factors) to phosphorylate PSF. Full-length His-tagged versions of PSF, ASF/SF2, and nonO were separately transformed and expressed in *E. coli* either alone or together with Dsk1 or its human orthologue, SRPK1 (PSF^D or PSF^S, respectively, in Figs. 2A and B). The proteins were purified and then tested by western blotting with mAb3C5. The prototypic RS protein, ASF/SF2, when co-expressed with Dsk1, was strongly recognized by mAb3C5 (Fig. 2A, lane 7), indicating that no other eukaryotic components were required. Likewise, PSF, co-expressed with either Dsk1 (PSF^D, Fig. 2 A and B, lanes 5) or with SRPK1 (PSF^S, Fig. 2B, Lane 6) was bound by mAb3C5, whereas PSF expressed alone was not (Fig. 2A and B, lanes 4). On the other hand, p54^{trb} and nonO did not react with the SR phosphorylation-specific mAb3C5 when co-expressed with the kinase (Fig 2A, lane 3 and data not shown).

Our results indicate that in *E. coli*, SRPK1 or Dsk1 is sufficient to create a phospho-epitope(s) for PSF that can be recognized by mAb3C5. However, we observed significantly reduced intensity for PSF relative to ASF/SF2 (Fig. 2A) under conditions of equal protein loading (data not shown). This indicates that the interaction between PSF^D and this monoclonal antibody is weaker than that of ASF/SF2. This result might reflect the scarcity of reactive RS dipeptides within PSF or that the fraction of PSF phosphorylated is less. The faster migrating band of PSF of 68 kD, which was routinely recognized by both anti-PSF and mAb3C5 (Fig. 2B and re-addressed below), is very likely a N-terminal-containing, proteolytically-truncated form of the same size that was previously reported in mammalian cells [14].

Taken with the results of Figs. 1, PSF appears to be a substrate for the SR kinases, Dsk1 and SRPK1, and the phosphorylation of PSF generates a conformational epitope similar to that found in conventional SR proteins.

PSF is phosphorylated by SRPK1 and Dsk1 in vitro

SR proteins are phosphorylated by human SRPK1 and fission yeast Dsk1 in a highly specific manner [4,41]. To determine whether PSF is a substrate of these two SR kinases, we performed *in vitro* phosphorylation assays. SRPK1 or Dsk1 was incubated with PSF, ASF/SF2, nonO or GST proteins separately. As with the conventional substrate ASF/SF2 (Fig. 3A and B, lanes 7), we found that PSF can be phosphorylated by either Dsk1 (Fig. 3A, lane 5) or SRPK1 (Fig. 3B, lane 5), albeit to a lesser extent. NonO and its human orthologue p54^{nrb} share high sequence identity with PSF, including an RSR tripeptide motif immediately N-terminal to the RRM. However, neither nonO (Fig. 3A and B, lanes 3) nor p54^{nrb} (data not shown) were phosphorylated by SRPK1 or Dsk1.

It was previously shown that autophosphorylation of Dsk1 is constitutive and occurs prior to substrate protein phosphorylation [41]. Autophosphorylation of SRPK1, on the other hand, is concentration-dependent [24]. Thus we suspect that the ~70kD band in lanes containing Dsk1 is autophosphorylated Dsk1. We suspect that the ~66kD band in Lane 7 of Fig.3B, as well as the very faint equivalently-sized band in other lanes that contain SRPK1, is either a bacterial contaminant, which accompanies SRPK1 purification, or a degradation product of SRPK1 that is particularly stable in the presence of the substrate ASF. Regardless, the results strongly indicate that PSF, but not its highly similar partner, p54^{nrb}/nonO, is an *in vitro* substrate of both SR kinases.

An isolated RS dipeptide near the N-terminus of PSF is the target of SR phosphorylation

PSF does not contain a conventional RS domain [4], but it contains 5 interspersed RS dipeptides. To test whether one or more of these is a genuine RS dipeptide responsible for the phosphorylation by SRPK1, we constructed GST-fusions of regions spanning each RS dipeptide, then expressed and purified each of them from *E. coli* (Fig. 4A). Only the N-terminal fragment (residues 1-18) was phosphorylated above background levels (Fig. 4B). Mutation of the RS dipeptide (residues 7 and 8 to LG) within the N-terminal region eliminated SRPK1 phosphorylation (Fig. 4C). That 7-RS-8 is the only site of SR phosphorylation was confirmed by the lack of reactivity of mAbC35 against truncation mutants (26-707 and 313-707) that lack this region but contain all other RS dipeptide candidates (data not shown). Thus, this isolated motif can convey SR kinase substrate specificity and anti-SR antibody immunological specificity to PSF.

We carried the experiment out on both the 7-RS-8 mutant and on No reactivity was found with the mAb. This further confirmed our conclusion that 7-RS-8 was the only site of SR phosphorylation in PSF. We have inserted this text as data not shown on p XX of the Results section.

SR Phosphorylation reduces the binding of PSF to the polypyrimidine tract

To test whether SR phosphorylation affects the ability of PSF to bind to its pre-mRNA target, we performed an *in vitro* gel mobility shift assay. In Fig 5A, bacterially produced non-phosphorylated PSF was incubated at increasing concentrations with a radiolabeled RNA oligonucleotide containing both the branch point and the Py (b/p+Py) from the intron of the Adenovirus major late (Adml) transcript [47]. With purified bacterially expressed protein, the slower migrating, more intense species corresponds to full-length PSF, while the lower species is likely the 68 kD breakdown product of Figs. 2B and 3 which was previously shown [14] to

bind to RNA. Both species show specific competition with cold bp+Py but not with a scrambled sequence competitor, NT3.

Having established specificity, we then tested equivalent concentrations (as judged by Commassie staining of inputs; data not shown) of non-phosphorylated and phosphorylated PSF (produced in and purified from *E. coli*) in the same assay (Fig. 5B). We found that PSF-RNA interaction was significantly reduced by SR-phosphorylation of PSF by either Dsk1 or SRPK1.

While there is considerable degradation of full length PSF, we have repeated this experiment several times with independent preparations of phosphorylated and nonphosphorylated PSF and obtained the same results (data not shown). If a degraded version of PSF was giving rise to the minor (faster mobility) gel shifted band as suspected [14], this species shows the same binding and competition behavior as the slower mobility gel-shifted (presumably) full-length PSF-containing RNA-complex. In bacteria, SR phosphorylation (as judged by the data of Fig. 2) and other experiments (eg, protein yields following Ni-resin purification; data not shown) appear to stabilize PSF relative to the nonphosphorylated form. Phosphorylated preparations of PSF undoubtedly contain nonphosphorylated substrate. Therefore, we suspect that the inhibitory effects of SR-phosphorylation of PSF observed in Fig.5B are an underestimate. We conclude that SR-phosphorylation of PSF regulates its ability to bind to the polypyrimidine tract of pre-mRNA introns.

DISCUSSION

When over-expressed in mammalian cells, PSF appears to be phosphorylated by an unidentified SR kinase, likely one of the SRPK family, such that an SR phosphorylation-specific monoclonal antibody (mAb3C5) could recognize it. These results were confirmed with an additional anti-SR monoclonal antibody, mAb104, for PSF expressed by baculoviral infection of insect cells (data not shown). We found that recombinant SRPK1 or Dsk1, SR kinases shown to have high specificity for their substrates [24,41], phosphorylate PSF *in vitro*. Furthermore, PSF is a substrate for both SR kinases when co-expressed with them in bacteria, as evidenced by their recognition with mAb3C5. On the other hand, neither nonO nor its human homologue, p54^{nrB}, which share high sequence identity/similarity with PSF, is phosphorylated under the same conditions. These results provided strong evidence that PSF is a non-classical substrate of SR kinases and that the phosphorylation is specific for RS dipeptides [24,41]. That conclusion was confirmed by mutagenesis experiments that demonstrated SR phosphorylation near the N-terminus at position 8.

PSF is an atypical SR substrate in that it contains no obvious RS domain. While unusual, this is not unprecedented. In budding yeast, Npl3p was reported to be phosphorylated by mammalian SRPK1 and by its yeast homolog Sky1 [26]. As with Np13p, in which only 1 of 8 RS dipeptides was phosphorylated [27], only 1 of 5 RS dipeptides was phosphorylated in PSF by SRPK1. According to Wang et al. [28], a serine (position 0) with arginines on both sides (positions -1 and +1), and with an arginine in position -3, is the preferred substrate for SR kinases [28]. In PSF, 7-RS-8 fits the consensus precisely (Fig. 4A), and this motif explains why only RS (1-18), out of 5 candidates was phosphorylated by SRPK1. On the other hand, p54^{nrB}/nonO contains an RS dipeptide, RSR, just N-terminal to its RNA recognition motif [9,10]. Perhaps it cannot be phosphorylated by SRPK1 because it lacks either an arginine at position -3 or the other criterion of Wang et al. [28], a basic path surrounding the targeted serine. We noted that the level of phosphorylation of the N-terminal, RS-containing fragment was weaker than that observed for full-length PSF. We suspect that this short fusion fragment might not be folded as natively as the full-length protein. It is also possible that other SRPK1 contacts presented in full-length PSF were lost in the deleted version.

PSF is an hnRNP protein that associates with RNA and snRNP complexes at multiple stages. For example, PSF binds to both U5snRNA [12] and the U4/U6.U5 tri-snRNP [13]. Since PSF also accumulates in the spliceosome complex B [6], it likely participates in bridging this complex with tri-snRNP. PSF interacts with the 5' splice site [48], but in addition, appears to accumulate in complex C where it could participate in the second step of catalysis [14]. Therefore, we felt it would be informative to determine the effects of SR phosphorylation of PSF on RNA binding. To address this, we chose to determine the functional consequence of SR phosphorylation on the binding of PSF to the intronic polypyrimidine track (Py). We observed a significant and specific reduction of Py binding by phosphorylated PSF. This is similar to what was observed for the non-conventional SR protein Np13, in which phosphorylation decreased its association with poly (A)⁺ RNA [35]. Our binding experiment of Fig. 5B indicates that the extent of phosphorylation correlates with the degree of inhibition. However, we cannot distinguish whether phosphorylation eliminates binding completely or whether it reduces binding affinity, because the preparations of phosphorylated PSF most probably contain unphosphorylated substrate. The same caveat holds for the conclusions drawn on Np13 [35]. In addition it was shown that modulation of Sky1-mediated SR phosphorylation of Np13 is required for its proper nuclear localization [27].

SR phosphorylation of conventional substrates, such as ASF/SF2, can alter their interactions with RNA or with other proteins to affect pre-mRNA processing and mRNA export [49-51]. Indeed, a phosphorylation-dephosphorylation cycle is necessary for splicing [52-54]. PSF has been shown in two-hybrid assays to interact with protein phosphatase type 1 (PP1) [55]. Microcystin-LR inhibits PP1 and PP2A by irreversibly binding to their catalytic subunits, thereby blocking the catalytic function of the spliceosome and the assembly of complexes B and C [55]. Both these complexes have been shown to contain PSF [6,14]. The finding that SRPK1, co-localizes in speckles with the splicing factors SC35 and B1C8 [56], indicates that an SR kinase, PSF and a putative phosphatase capable of reversing the reaction are present in the same place at the same time. SR proteins have been reported in *Drosophila* for regulating alternative splicing and sex determination, and phosphorylation by *Doa*, a LAMMER kinase, plays an important role in this regulation [57].

While PSF can serve as a substrate for SRPK1, our data do not address whether PSF is phosphorylated exclusively by SRPK1 in mammalian cells. PSF was reported to be hyperphosphorylated on serine and threonine residues during apoptosis—an event that dissociates PSF from PTB, leading to binding interactions with new partners [58]. It remains to be determined whether differential phosphorylation of PSF plays a role in these events. Further investigation is necessary to determine additional functional consequences of unconventional SR phosphorylation of PSF.

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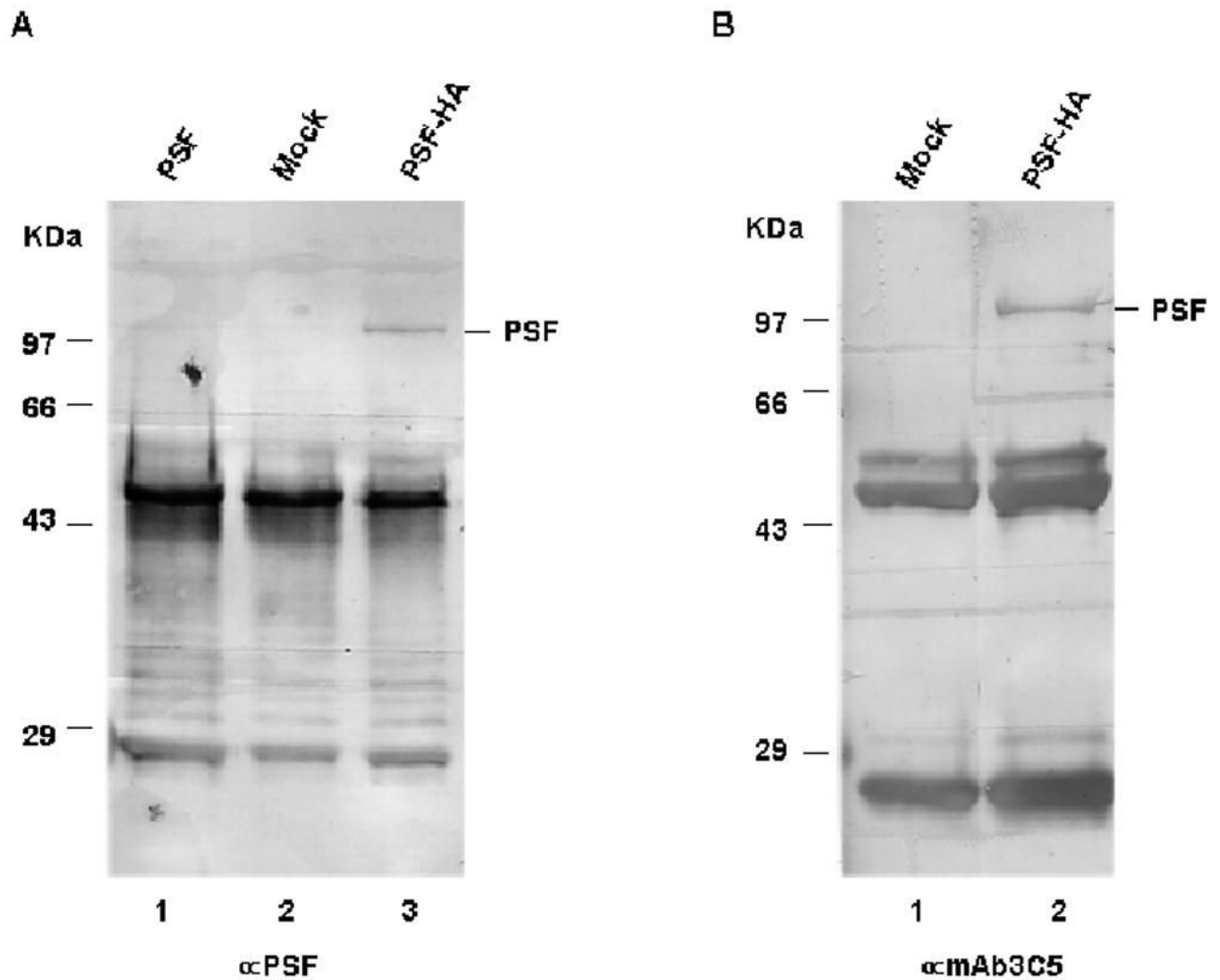
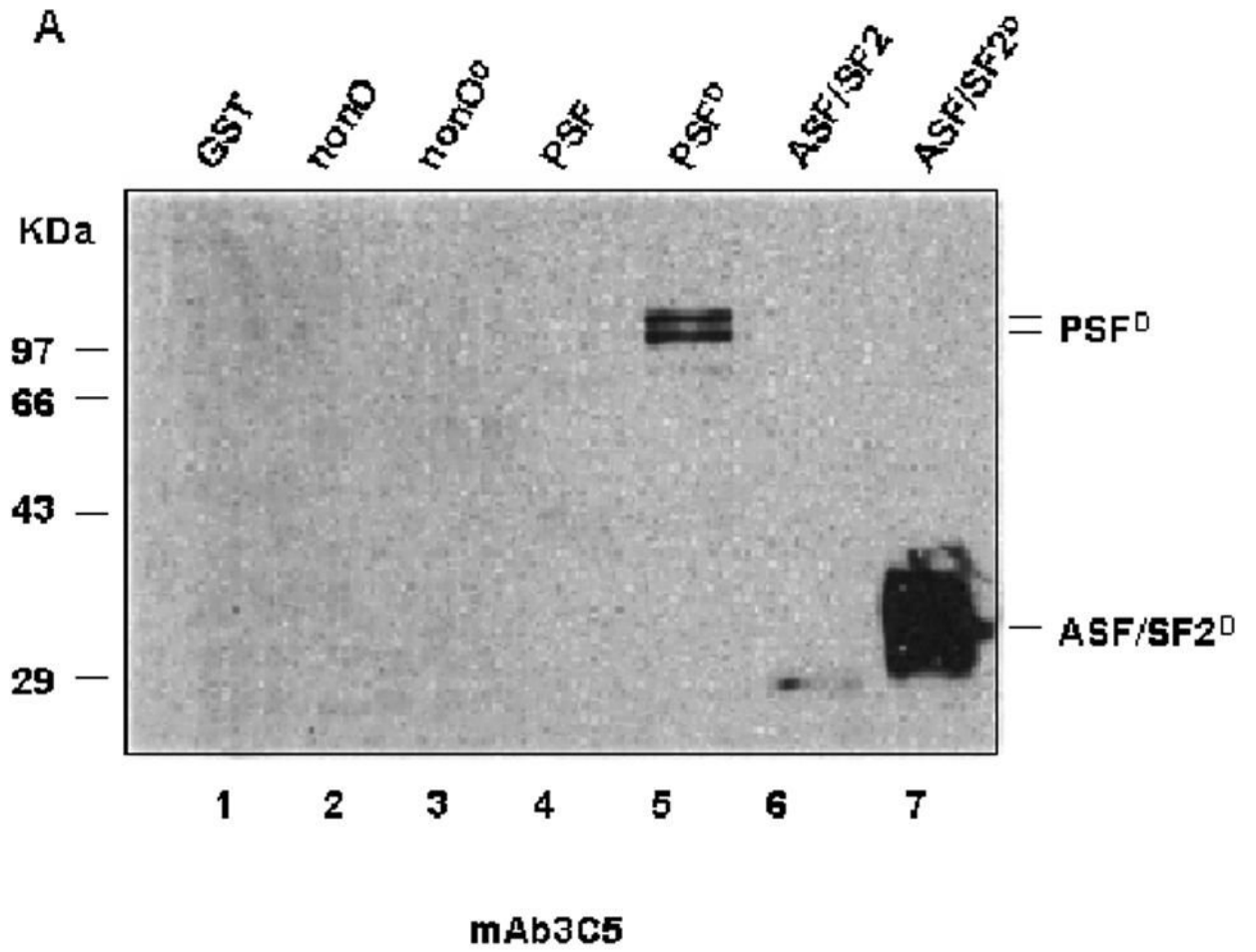


Figure 1.

PSF can be recognized by SR phosphorylation-specific mAb 3C5 in mammalian cells.

HA-tagged PSF (pCR3.1-PSF-HA), untagged PSF (pCR3.1-PSF) or the empty expression vector (mock) was transiently transfected into Cos-7 cells. After 48 hrs, supernatants from whole cell lysates were collected and immunoprecipitated with an anti-HA monoclonal antibody. Samples were resolved on 10% SDS-PAGE, transferred to a PVDF membrane, and then western blotting was performed with anti-PSF (A) or mAb 3C5 (B). Bands at ~50 and 25 kD in all samples result from reaction of the anti-mouse secondary antibody against heavy and light immunoglobulin chains.



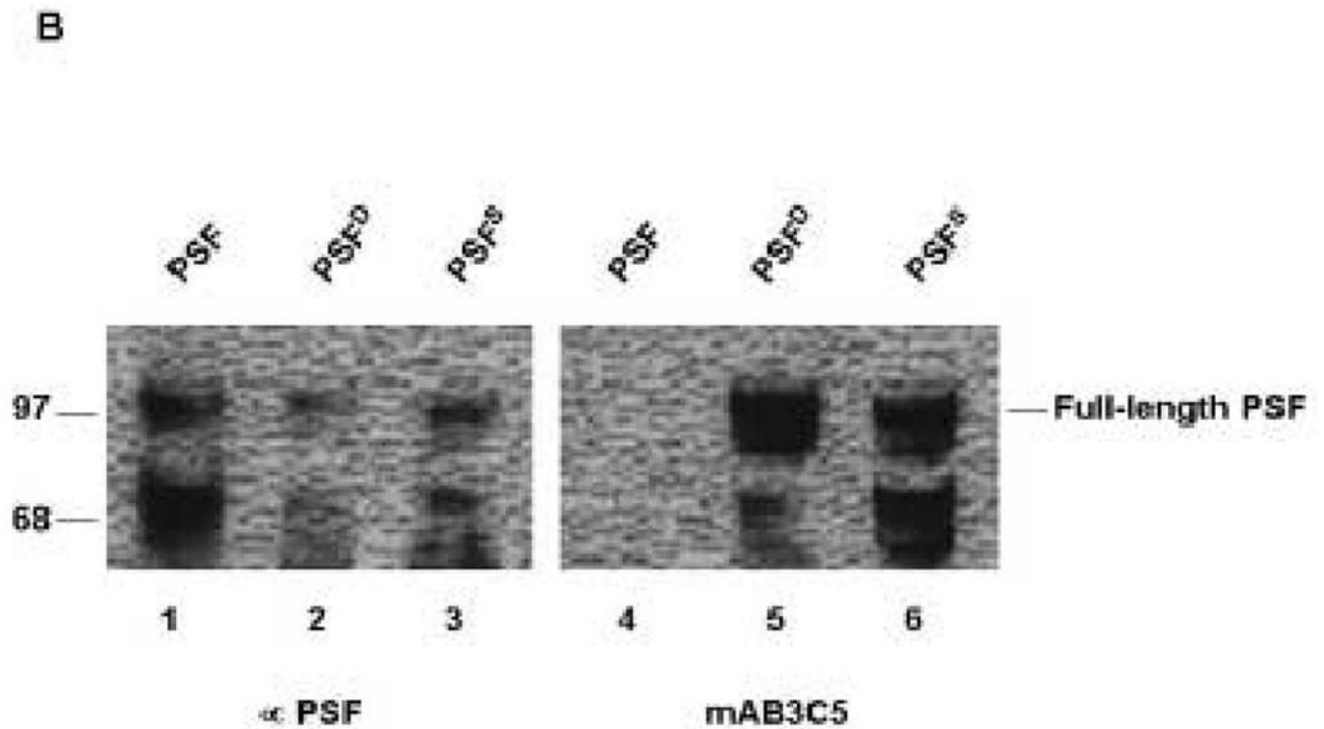


Figure 2.

PSF co-expressed with SR kinases in bacteria can be recognized by SR phosphorylation-specific mAb3C5. (A). 2 μ M of GST, or His-tagged proteins (nonO, PSF or ASF/SF2) or the same proteins co-expressed with Dsk1 (nonO^D, PSF^D or ASF/SF2^D) were resolved by 10% SDS-PAGE, transferred to PVDF membranes, and then western blotted with mAb3C5. (B). 2 μ M of purified PSF alone or PSF co-expressed with Dsk1 (PSF^D) or SRPK1 (PSF^S) were separated as above and filters were blotted with anti-PSF (left panel) or mAb3C5 (right panel). The reactive species at 68 kD is a proteolytic fragment of PSF previously observed at high abundance [14].

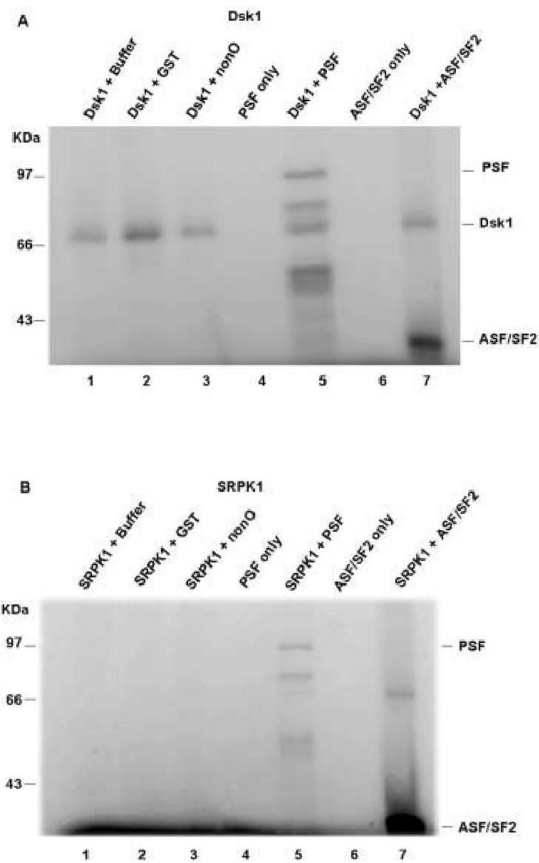
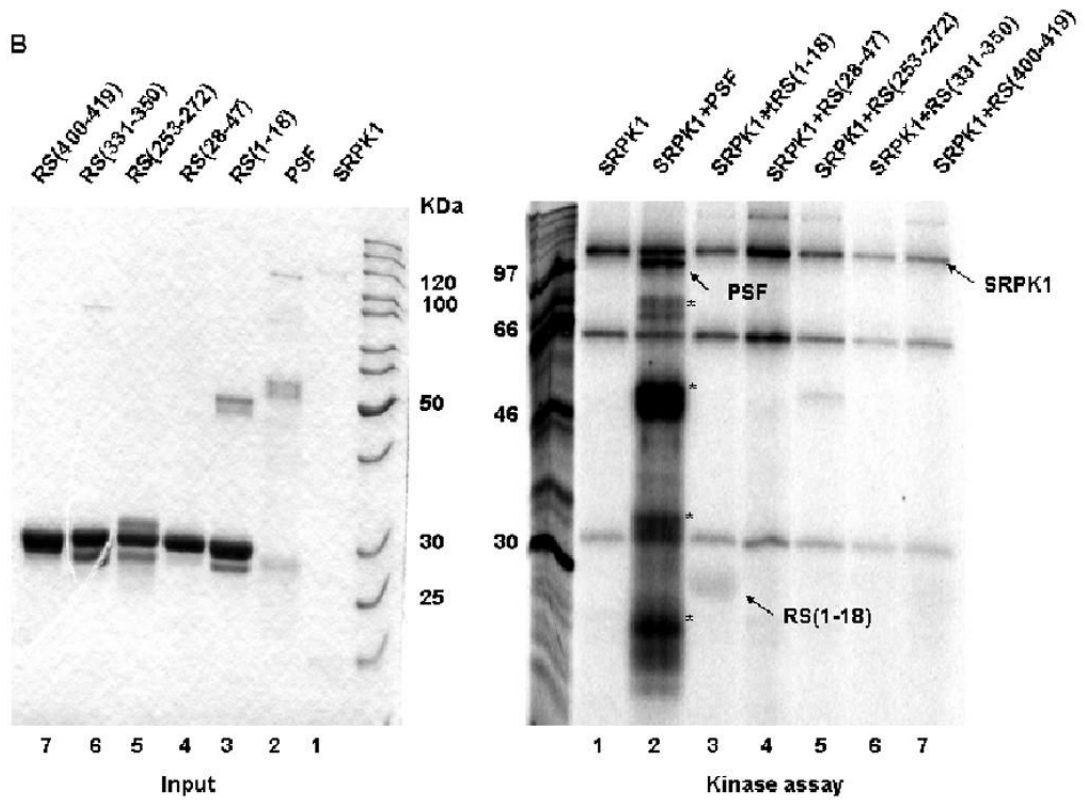


Figure 3.

***In vitro* phosphorylation of PSF by SRPK1 or Dsk1.** 1 μ M of the indicated bacterially expressed and purified recombinant proteins were incubated with 0.5 μ M Dsk1 (A) or with the same concentration of SRPK1 (B) in the presence of [γ - 32 P]ATP at 23°C for 30 min. Samples were resolved by 10% SDS-PAGE and visualized by phosphoimaging. Positions of phosphorylated PSF and autophosphorylated kinase are indicated.

1-MSRDRFRSRGGGGGGFHR-18	RS(1-18)
1-MSRDRFLGRGGGGGGFHR-18	RS(1-18), RS-LG
28-LHDFRSPPPGMGLNQNRPGM-47	RS(28-47)
253-QQHHQGPPGGPGGRSEEKI-272	RS(253-272)
331-GKGFQFIKLESRALAEIACA-350	RS(331-350)
400-AVVIVDDRGRSTGKGIVEFA-419	RS(400-419)



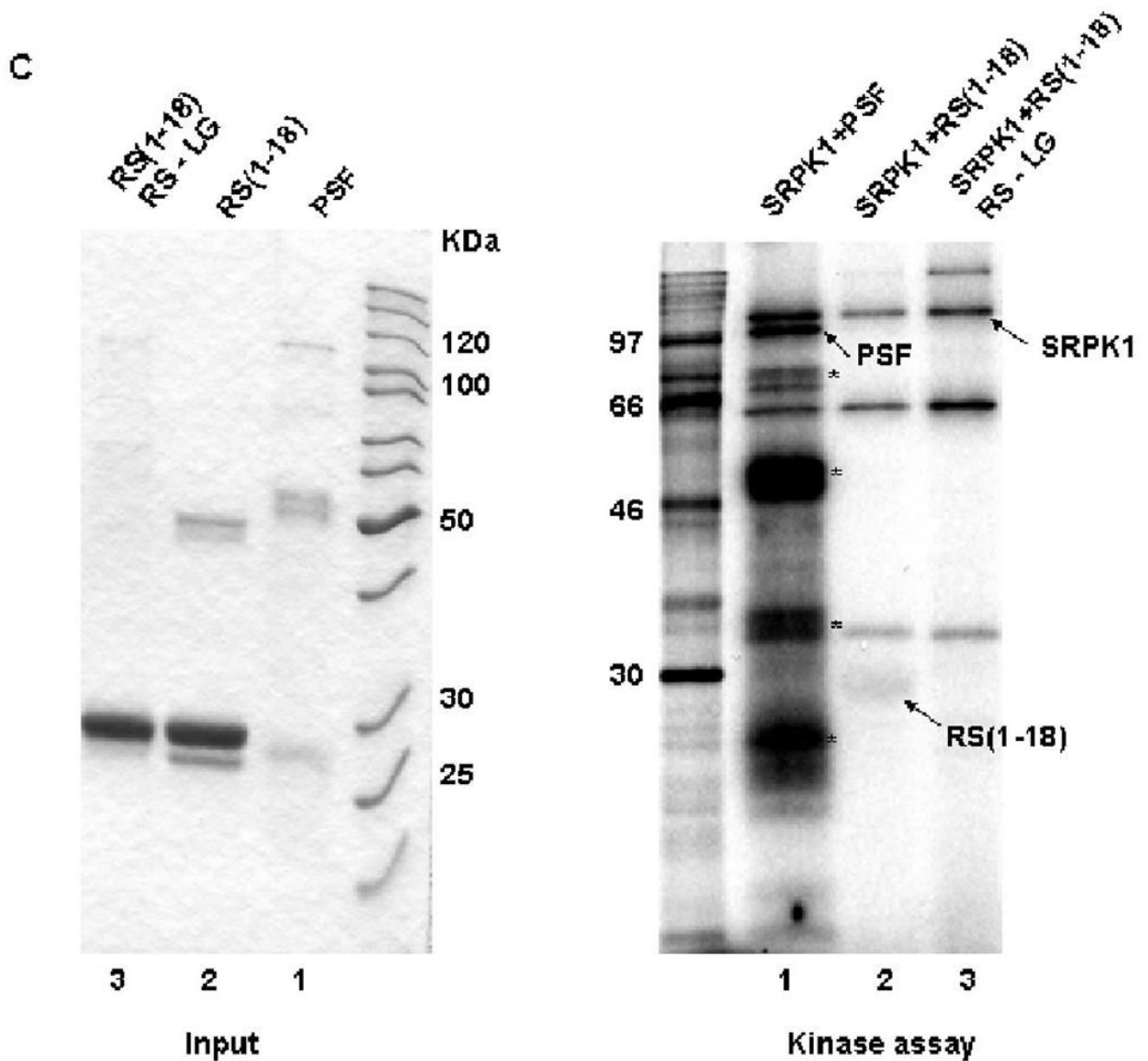
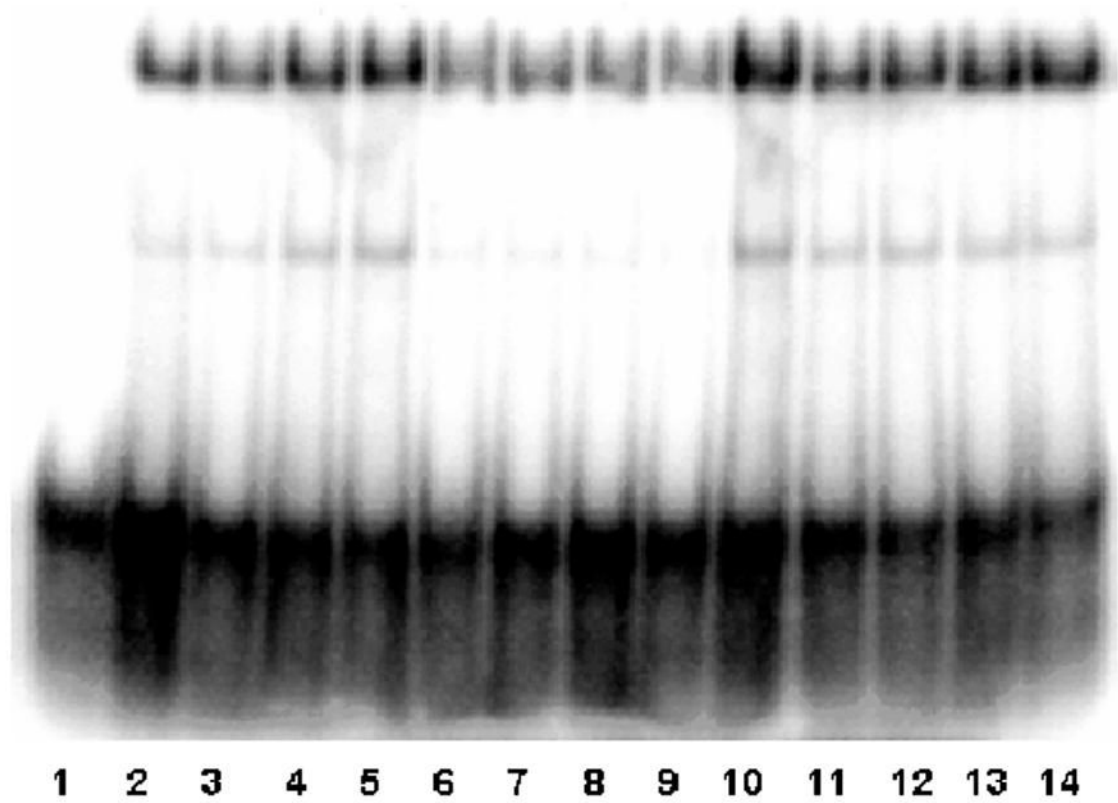


Figure 4.

An N-terminal RS dipeptide within PSF is phosphorylated by SRPK1. (A). Amino acid sequences spanning the five RS dipeptides, the two mutations of 7-RS-8 within the N-terminal (1-18) region, and their relative positions within PSF. All peptides were expressed and purified as GST fusion proteins. (B) The indicated bacterially expressed GST fusion proteins were incubated with SRPK1 in the presence of [γ - 32 P]ATP at 23°C for 30 min (right panel). Integrity and equal inputs for each fusion protein were confirmed by Commaise staining (left panel). (C) Bacterially expressed RS(1-18) wildtype and mutated peptides (left panel) were incubated with SRPK1 in the presence of [γ - 32 P]ATP at 23°C for 30 min (right panel). The samples were resolved on 10% SDS-PAGE and visualized by phosphoimaging. Asterisks denote degradation products of full-length PSF. The apparent increase in signal of SRPK1-mediated phosphorylation in this experiment relative to that in Fig. 3B results from the use of a higher specific activity 32 P-ATP substrate

A

PSF	0	0.5	1.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	(μM)
b/p+py	—	—	—	—	—	50	100	150	200	—	—	—	—	—	(ng)
control	—	—	—	—	—	—	—	—	—	—	50	100	150	200	(ng)



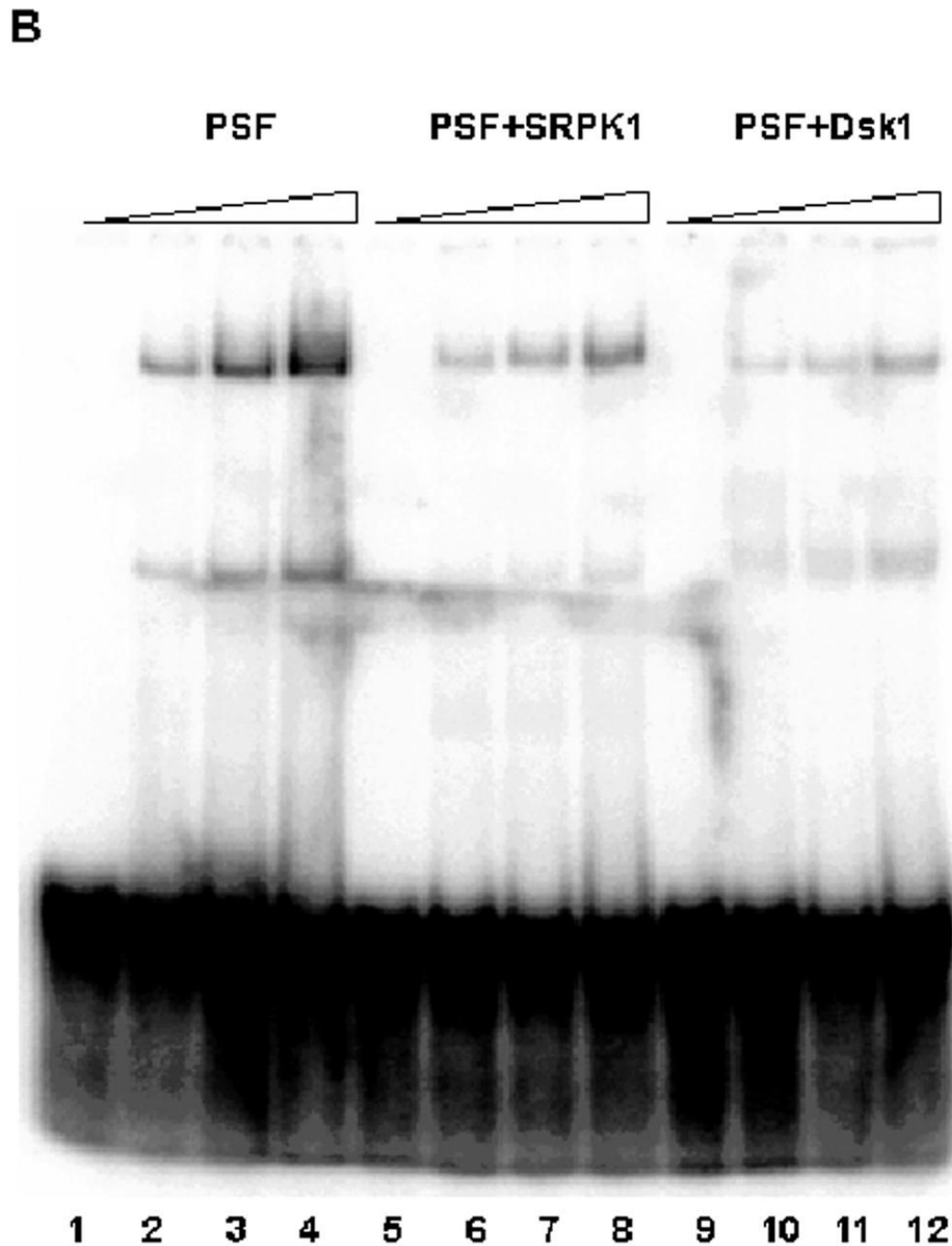


Figure 5.

SR phosphorylation regulates the *in vitro* binding of PSF to the polypyrimidine tract of the 3' splice site. (A) Specificity of PSF-pyrimidine (py) tract binding. PSF expressed alone in and purified from E coli was incubated with a labeled RNA oligo (~1ng) containing the branch point (bp) and py (bp+py) at 30°C for 25 min (lane, 1-4). Specific (lanes 5-9) or non-specific (lanes 10-14) unlabeled competitors were added at varying concentrations to the binding reactions prior to resolution on a 6% native polyacrylamide gel and visualization by phosphoimaging. (B) SR phosphorylation reduces binding. PSF (lanes 1-4) or PSF co-expressed with Dsk1 (PSF^D) (lanes 5-8) or SRPK1 (PSF^S) (lanes 9-12) were incubated

separately with the labeled RNA bp+py-containing oligo (~1ng) at 30°C for 25 min. Samples were resolved and visualized as in(A).