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Identification of a novel PSR as the substrate of an SR protein kinase in the true slime mold

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Here, a novel cDNA encoding a serine/arginine (SR)-rich protein, designated PSR, was isolated from the true slime mold *Physarum polycephalum* and expressed in *Escherichia coli*. The deduced amino acid (aa) sequence reveals that PSR contains RS repeats at its C-terminus, similar to the conventional PSRPK substrate ASF/SF2. To study the novel protein, we generated a variety of mutant constructs by PCR and site-directed mutagenesis. Our analysis indicated that the purified recombinant PSR was phosphorylated by PSRPK *in vitro* and the SR-rich domain (amino acids 460–469) in the PSR protein was required for phosphorylation. In addition, removal of the docking motif (amino acids 424–450) from PSR significantly reduced the overall catalytic efficiency of the phosphorylation reaction. We also found that the conserved ATP-binding region ^{62}LGWGHFSTVWLAIDE KNGGREVALK^{86} and the serine/threonine protein kinases active-site signature ^{184}IIHTDLKPENVLL^{196} of PSRPK played a crucial role in substrate phosphorylation and Lys^{86} and Asp^{188} were crucial for PSRPK phosphorylation of PSR. These results suggest that PSR is a novel SR-related protein that is phosphorylated by PSRPK.

Keywords: phosphorylation/Physarum polycephalum/PSRPK/SR-related protein.

Abbreviations: ASF, alternate splicing factor; RRM, RNA recognition motif; SF2, splicing factor 2; snRNPs, small nuclear ribonucleoprotein particles; SR protein, splicing factor containing arginine-serine repeats; SRPK, SR protein kinase; wt, wild-type.

Serine/arginine (SR)-rich proteins are highly conserved in metazoans and have a unique domain architecture consisting of at least one RNA recognition motif (RRM) at the N-terminus and an extensively phosphorylated domain that is rich in long stretches of RS dipeptides (RS domains) at the C-terminus. The RRMs are important for sequence-specific binding that commits pre-mRNA to the splicing pathway (*1*), whereas RS domains primarily function in protein–protein interactions with other SR proteins. SR domains also participate in the targeting of the proteins to speckles and may be important for the structural integrity of these subnuclear structures (*2–4*).

It is well-known that SR proteins coordinate protein–protein interactions through their RS domain and recruit other splicing components (*5*). For example, SR proteins interact with exonic splicing enhancer (ESE) sequences (*6*) and with the intronic branchpoint (*7*). ASF/SF2, an SR protein that promotes the interaction of mRNA and the U1-70 K RNP protein, plays an important role in the recognition of the 5′-splice site and is essential for the first cleavage reaction during pre-mRNA splicing (*8*). In addition to these early roles, SR family proteins are also known to connect spliced mRNA to the RNA export machinery and to modulate protein translation in the cytoplasm (*9–11*). The RS domains of SR proteins are extensively phosphorylated. Clk and SRPK, two groups of kinases, are believed to be largely responsible for SR protein phosphorylation (*12*, *13*). Mammalian SR protein kinase 1 (SRPK1) and the yeast enzyme Sky1p are the two of the most-studied SRPKs. Members of the SRPK family display strict substrate specificity, preferring to phosphorylate only serine residues flanked by arginines. ASF/SF2 is a well-studied mammalian SR protein that contains 20 serines within its 50-residue long SR domain. SRPK1 phosphorylates 10–12 of these serines in the N-terminal RS1 region of the SR domain (*14*). This multi-site phosphorylation occurs via a processive mechanism in which the enzyme stays attached for about eight cycles of rapid serine phosphorylation before releasing the splicing factor (*14*, *15*). SRPK1 phosphorylates ASF/SF2 by an ordered mechanism in which the kinase initiates phosphorylation at the center of the SR domain near the SR1/SR2 boundary and attaches phosphates onto serines that are moving in a general C-terminal to N-terminal direction (*16*).

Members of the SR protein family are highly conserved during evolution. The SR family of proteins comprises a group of at least eight evolutionarily conserved members, including SRp20, SRp30a/ASF/SF2, SRp30b/SC35, SRp30c, 9G8, SRp40, SRp55 and SRp75 (*1*, *17*, *18*). Among higher eukaryotes, many members of the SR protein family have been identified and characterized, but only a limited number of SR protein genes have been reported in lower eukaryotes such as *Physarum polycephalum*.

Physarum polycephalum is among the well-characterized plasmodial slime molds (Myxomycetes) that typically have a life cycle involving haploid (spores, amoebae) and diploid (plasmodia) cell forms. Together with the cellular slime mold *Dictyostelium discoideum* and other Mycetozoa, *P. polycephalum* has been classified among the multi-cellular lower eukaryotes on the basis of molecular phylogenetic criteria (*19*). In a previous study, we isolated the cDNA of an SRPK-like protein from *P. polycephalum*, designated as PSRPK (accession no. DQ140379). Similar to other SRPKs, PSRPK has two conserved domains and can phosphorylate human SR, indicating that PSRPK is a novel member of the SRPK family (*20*). In this study, we isolated a gene encoding the PSR protein, a novel SR-related protein, from *P. polycephalum*. We further characterized PSR by evaluating the ability of PSRPK to phosphorylate this protein as well as the effects on this phosphorylation event by deletion or mutation of the key functional domains on PSRPK and PSR.

Materials and Methods

Materials

The strain TU291 of the true slime mold *P. polycephalum* was kindly provided by Dr Philipe Albert (Cytobiology Laboratory, Reims University, France). Microplasmodia was cultivated as described previously (*21*). [γ-^{32}P] ATP was obtained from Bio Basic Inc. (Toronto, Canada).

Isolation of PSR cDNA

A Matchmaker Gal4 Two-hybrid System (BD Clontech Laboratories, Inc.) was used for the two-hybrid screening following the manufacturer's instructions. A 2×10^6 transformants/3 μg pGADT7-Rec of transformation efficiency, 5.35×10^8 cells/ml of cell density and 2.34×10^9 cfu/ml titre of *P. polycephalum* two-hybrid AD library was constructed (*22*). About 2×10^6 yeast transformants were screened with the plasmid pGBKT7-14-3-3d (bait) following the commercial instruction (*23*). Positive clones were selected and assayed for β-galactosidase activity using filter-lift assay. The positive candidates were sequenced and analysed by nucleotide comparison to sequences within GenBank using BLAST software (NCBI).

The 483-bp cDNA fragment of PSR was screened from a cDNA library of *P. polycephalum*. In order to isolate the full-length PSR cDNA, total RNA was isolated from amoebae and plasmodia of *P. polycephalum* using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). The first-strand cDNA was prepared by random priming from 5 μg of total RNA using a GeneRacer^{TM} Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Degenerate hybrid primers were then mixed with the reverse transcripts and denatured at 94°C for 180 s, followed by five polymerase chain reaction (PCR) cycles at 94°C for 30 s and 72°C for 150 s. To isolate the 5′-end of the cDNA fragment of pSR, 5′-RLM-rapid amplification of cDNA ends (5′-RLM-RACE) was performed according to the manufacturer's instructions. The primers for walking in the 5′-direction were as follows: 5′-GGACACTGAC ATGGACTGAAGGAGTA-3′ (forward) and 5′-CTCCCGCAATC GGCAACCCAATAGC-3′ (reverse). The PCR conditions were as follows: 94°C for 180 s followed by 30 cycles at 94°C for 30 s, 65°C for 30 s and 68°C for 150 s. The full-length clone was obtained using primers 5′-CGGGATCCATGTATTCACGTTGTATCGCTCTTG -3′ (forward) and 5′-ACGCGTCGACTTAGTCTGAATCCGAAT CTCTTGAG-3′ (reverse). Both strands were confirmed by complete sequencing.

Plasmid construction and site-directed mutagenesis

Wild-type (wt) PSR and its truncated mutants tpN1 (amino acids 1–424), tpN2 (amino acids 1–460), tpC1 (amino acids 460–497), tpC2 (amino acids 451–497), tpC3 (amino acids 434–497), tpC4 (amino acids 424–497) and tpC5 (amino acids 338–497) were amplified from the cDNA library of the plasmodia of *P. polycephalum* using the primers listed in Table I. The amplified fragments were cloned into the *BamH*I and *EcoR*I sites of the pET-32a (+) expression vector (Novagen, Madison, WI, Germany). The site-directed mutagenesis of the PSR gene was performed by PCR amplification using the *Pfu* DNA polymerase (Fermentas, ON, Canada). PSR-mRK contained one Arg to Lys point mutation at position 464 and PSR-mST contained one Ser to Thr point mutation at position 465 in the SR domain.

The pGEX-PSRPK plasmid was previously described (*20*). To generate the truncated mutants PSRPK-dABR and PSRPK-dAS, the cDNAs were amplified by PCR using the primers listed in Table II, digested with *EcoR*I/*Sal*I and ligated into the *EcoR*I/*Sal*I sites of pGEX-5X-1(Amersham Biotech, Uppsala, Sweden). PSRPK-mKM contained one Lys to Met point mutation at position 86. PSRPK-mDA was constructed by replacing Asp at position 188 with Ala. All mutant constructs were confirmed by DNA sequencing.

Expression and Purification of Recombinant Proteins

Expression and purification of His-ASF/SF2 and GST-PSRPK were carried out as previously described (*20*). The cDNAs encoding PSR and its truncated mutants were amplified and inserted into pET-32a (+) (Novagen), and the recombinant plasmids were transformed into *Escherichia coli* strain BL21 (DE3). The cultures were grown at 37°C in 21 of LB broth containing 100 μg/ml ampicillin. Protein expression was induced in these cells with 0.1 mM isopropyl β-D-thiogalactoside (IPTG) at 37°C for 16 h before collection by centrifugation.

Table I. Primers used for cloning *PSR* and truncated *PSR*.

Sequence	Sequence of forward and reverse primers	Restriction sites	PCR product size (bp)
pSR	F-5′-CGGGATCCATGTATTCACGTTGTATCGCTCTTG-3′	BamH I	1494
(1–497 amino acids)	R-5′-ACGCGTCGACTTAGTCTGAATCCGAATCTCTTGAG-3′	Sal I	
tpC1	F-5′-CCGGAATTCCGATCACGTTCCAGGAGTAG -3′	EcoR I	117
(460–497 amino acids)	R-5′-ACGCGTCGACGTCTGAATCCGAATC-3′	Sal I	
tpC2	F-5′-CCGGAATTCCGATCTGGGCAGTCTG-3′	EcoR I	144
(451–497 amino acids)	R-5′-ACGCGTCGACGTCTGAATCCGAATC-3′	Sal I	
tpC3	F-5′-CCGGAATTCGACATGAGAGATGATGAC-3′	EcoR I	195
(434–497 amino acids)	R-5′-ACGCGTCGACGTCTGAATCCGAATC-3′	Sal I	
tpC4	F-5′-CCGGAATTCATGGGCAGTAAAACG-3′	EcoR I	225
(424–497 amino acids)	R-5′-ACGCGTCGACGTCTGAATCCGAATC-3′	Sal I	
tpC5	F-5′-CCGGAATTCATGCCATTTTGTACATCTGG-3′	EcoR I	483
(424–497 amino acids)	R-5′-ACGCGTCGACTTAGTCTGAATCCGAATCTC-3′	Sal I	
tpN1	F-5′-CGGGATCCATGTATTCACGTTGTATCGC-3′	BamH I	1296
(1–423 amino acids)	R-5′-ACGCGTCGACGTGTTTCCTCAATAATTC-3′	Sal I	
tpN2	F-5′-CGGGATCCATGTATTCACGTTGTATCGC-3′	BamH I	1377
(1–459 amino acids)	R-5′-ACGCGTCGACGCCCAGATCGCCCAGACT-3′	Sal I	

The highlights represent the sequences of endonuclease sites.

Table II. Primers used for cloning mutants and deletions of truncated *PSRPK*.

Name	Sequence of forward and reverse primers	Size (bp)
psprk-mKM	F-5′-CGGGAAGTAGCGTTAA[TG]ATAGTAAAAAGTG-3′	1284
(86 K→M)	F-5′-CACTTTTTACTAT[CA]TTAACGCTACTTCCCG-3′	
psrpk-dABR	F-5′-CAGAATCGTAAAGAAAATAGTAAAAAGTGCATC-3′	1209
(deletion 62–86 amino acids)	F-5′-GATGCACTTTTTACTATTTTCTTTACGATTCTG-3′	
psrpk-mDA	F-5′-ATTATACACACGG[C]CCTCAAACCTG-3′	1284
(465 S-to-T)	F-5′-CAGGTTTGAGG[G]CCGTGTGTATAAT-3′	
psrpk-dAS	F-5′-CTTACACACAAAATGCAAAGACCATCTGTTACGAC-3′	1245
(deletion 184–196 amino acids)	F-5′-GTCGTAACAGATGGTCTTTGCATTTTGTGTGTAAG-3′	

The boxed sequences indicate mutated nucleotides.

The cells were then washed with PB buffer [20 mM Hepes (pH 7.5), 150 mM MgCl, 1 mM DTT, 0.5 mM EDTA and 0.05% Nonidet P-40)] and suspended in 1 ml of ice-cold PB. The pelleted cells were lysed by sonication in 150 ml of lysis buffer [50 mM NaCl/20 mM Mes (pH 6.5)/20% (vol/vol) glycerol/1 mM PMSF/0.5 ml of protease inhibitor mixture (Sigma, St Louis, MO, USA)] (10 × 15 s bursts with 5 s for cooling between bursts) and then centrifuged for 30 min. Strains expressing the GST-fusion proteins were cultured and induced in the same manner.

All purification steps were carried out at 0–4°C. The His-tagged PSR protein was purified by affinity chromatography using Ni^+ beads according to the manufacturer's protocol (Amersham Biotech, Uppsala, Sweden). The lysate was centrifuged for 20 min to separate the supernatant and cell debris. The supernatant was incubated with Ni^+ beads for 1.5 h, after which the beads were spun down and washed three times with 200 µl elution buffer [50 mM Tris–HCl (pH 8.0), 100 mM NaCl, and 50 mM imidazole] for 20 min and then rinsed twice with 1 ml 50 mM Tris–HCl (pH 7.4). The purified proteins were analysed by 10% sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE), followed by staining with 0.1% Coomassie Blue. The protein concentrations were determined by the Bradford assay (Bio-Rad Protein Assay) according to the manufacturer's instructions.

Phosphorylation assay in vitro

The reaction mixtures for protein kinase activity contained equal amounts (1 µM) of each recombinant wt pSR and mutants, 50 ng of bacterially expressed PSRPK in a kinase buffer [50 mM Tris–HCl buffer (pH 7.4), 10 mM $MgCl_2$, and 1 mM dithiothreitol] and 2 µCi of [γ-^{32}P] ATP in 25 µl final volume and were incubated at 30°C for 30 min. The reactions were terminated by boiling in a SDS–PAGE sample loading buffer. Proteins were separated by SDS–PAGE, transferred to nitrocellulose membrane and then exposed to X-ray film by autoradiography.

Results

Full-length cDNA cloning and molecular characterization of PSR

A partial cDNA encoding an SR-related protein was obtained from the *P. polycephalum* cDNA library by yeast two-hybrid screening. A 1494-bp full-length PSR ORF cDNA (accession no. FJ917746) (Fig. 1) was obtained by using the 5′-RLM-RACE strategy and was determined to encode a protein of 497 amino acids. This protein, which has similarities to SR-related proteins, was designated PSR (Fig. 2).

Several SR proteins are presently known, including the well-studied human ASF/SF2 protein. Compared with human ASF/SF2 (accession no. NM006924), the PSR protein sequence has two regions of significant similarity: (*1*) PSR amino acids 449–475 versus ASF amino acids 201–225 (79.2%) and (*2*) PSR amino acids 426–436 versus ASF amino acids 183–194 (54.5%). Based on the primary amino acid sequence (Fig. 2),

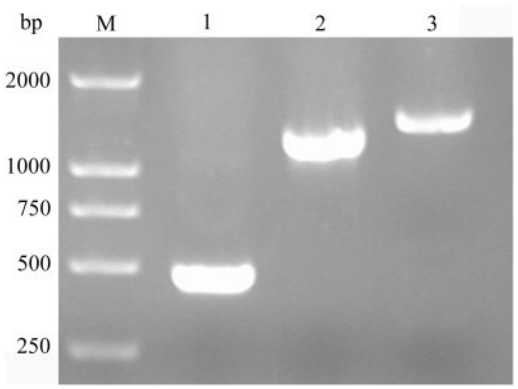

Fig. 1 Cloning of *PSR* cDNA from *P. polycephalum* by RT-PCR and 5′-RLM-RACE. Gene-specific primers were designed based on the nucleotide sequences of PSR. M: standard molecular weight DNA marker DL2000. Lanes 1–3: the PCR products of PSR 3′-cDNA (483 bp), PSR 5′-cDNA (1348 bp) and PSR ORF (1494 bp), respectively.

PSR contains contiguous SR dipeptides at the C-terminus (amino acids 460–469) and a docking motif near the C-terminal RS domain spanning amino acids 424–450. However, unlike the proteins of the SR family of splicing factors, it lacks an RRM motif, which is characteristic of some metazoan SR proteins (*24*). The difference between human ASF/SF2 and PSR could be due to the greater genetic divergence of these two species.

PSR is phosphorylated by PSRPK in vitro

SR proteins are extensively phosphorylated in a highly specific manner by kinases of the SRPK and Clk families (*3*, *4*). To determine whether PSR is phosphorylated by SR kinases, the purified PSR, ASF/SF2 were incubated with PSRPK (Fig. 3A). Following incubation in the presence of [γ-^{32}P] ATP, the two phosphorylated bands were observed in the autoradiographs (Fig. 3B). and as with the conventional substrate ASF/SF2 (Fig. 3B, lane 3), PSR could be phosphorylated by PSRPK *in vitro* (Fig. 3B, lane 1). This suggests that PSR is an endogenous substrate for PSRPK and indicates that PSR possesses the landmark biochemical characteristics of conventional SR proteins.

C-terminal RS dipeptides and the docking motif of PSR are required for its phosphorylation by PSRPK

To identify the RS domain required for phosphorylation by PSRPK, we first constructed a series of N-terminal

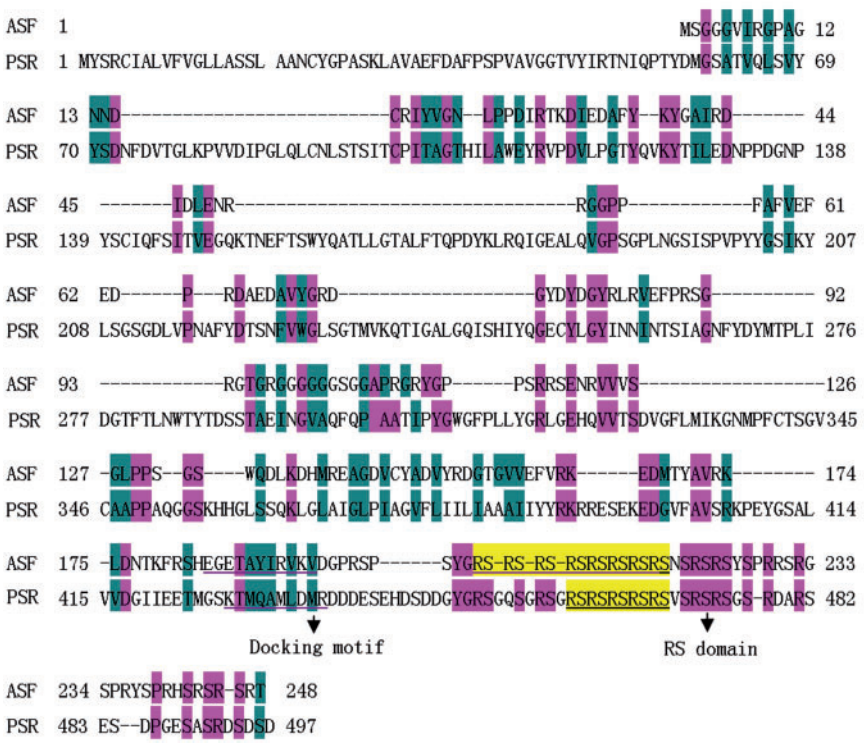

Fig. 2 Comparison of amino acid sequences of PSR and human ASF/SF2. Identical amino acids are indicated by red boxes. The docking motif and SR repeat domain are underlined.

and C-terminal deletions in the substrate (Fig. 4) and then expressed and purified each of them from *E. coli* (Fig. 5A). After incubation of equal amounts (1μM) of each purified mutant substrate with PSRPK in the presence of [γ-^{32}P] ATP, the fragments containing both the docking motif and RS domains tpC3 (amino acids 434–497), tpC4 (amino acids 424–497) and tpC5 (amino acids 338–497) were phosphorylated by PSRPK (Fig. 5B). We next generated a PSR mutant (dRS) with a deletion of the entire SR domain (amino acids 451–497) and expressed it in bacteria with an appended His tag (Fig. 6A, dRS lane). It was subsequently purified and incubated with recombinant PSRPK *in vitro*. Our results showed that while the wt protein was efficiently phosphorylated, the His-PSR (dRS) mutant protein remained unphosphorylated (Fig. 6B, dRS lane), suggesting that the SR segment is required for its phophorylation *in vitro*.

To determine whether the SR protein without the N-terminal Motif (NTM) could be phosphorylated by PSRPK, we constructed two deletion mutants, PSR (tpC4) and PSR (tpC5) (Fig. 4). These mutants were expressed and purified as His-fusion proteins in *E. coli* (Fig. 5A, lanes 8 and 9, Supplementary Data) for the phosphorylation assay. In the absence of all or part of the N-terminal peptides, PSR (tp C4) and PSR (tp C5) were phosphorylated to a slightly lower extent than wt PSR (Fig. 5B, lanes 7 and 8), suggesting that the peptide segment in the N-terminal half of PSR was not necessary for efficient phosphorylation of the SR domain. These results suggest that the docking motif and RS domain are necessary for the efficient phosphorylation of PSR.

The contiguous RS dipeptides near the C-terminus of PSR are the target of SR phosphorylation

SR proteins are extensively phosphorylated, mainly on the serine residues within the RS domain. To test whether the contiguous RS dipeptides near the C-terminus of PSR are involved in phosphorylation by PSRPK, site-directed mutagenesis was carried out within the RS domain of PSR. We mutated arginine residues to lysine at position 464 and serine residues to threonine at position 465 in the RS domain and then evaluated by autoradiography the phosphorylation of the resulting purified mutants, PSR (mRK) and PSR (mST), by a GST-PSRPK fusion protein *in vitro*. Mutation of the arginines (Fig. 6B, mRK lane) or serines (Fig. 6B, mST lane) within the RS domain of the RS dipeptide severely reduced its phosphorylation by PSRPK, indicating that the contiguous RS dipeptides near the C-terminus of PSR are the targets of SR phosphorylation.

The docking motif-like sequence of PSR is involved in its phosphorylation by PSRPK

Recently, it was shown that a docking motif in ASF/SF2 specifically interacts with a groove in SRPK1 and this interaction is necessary for processive phosphorylation (*25*). Comparison of the sequences of ASF/SF2 and PSR indicates that the peptide sequence present near the RS domain is homologous to the docking motif identified in ASF/SF2. To test whether the PSR docking motif is involved in kinase recognition, we deleted the entire putative docking motif [PSR (dDock), amino acids 424–450] and analysed the phosphorylation efficiency of the mutants compared

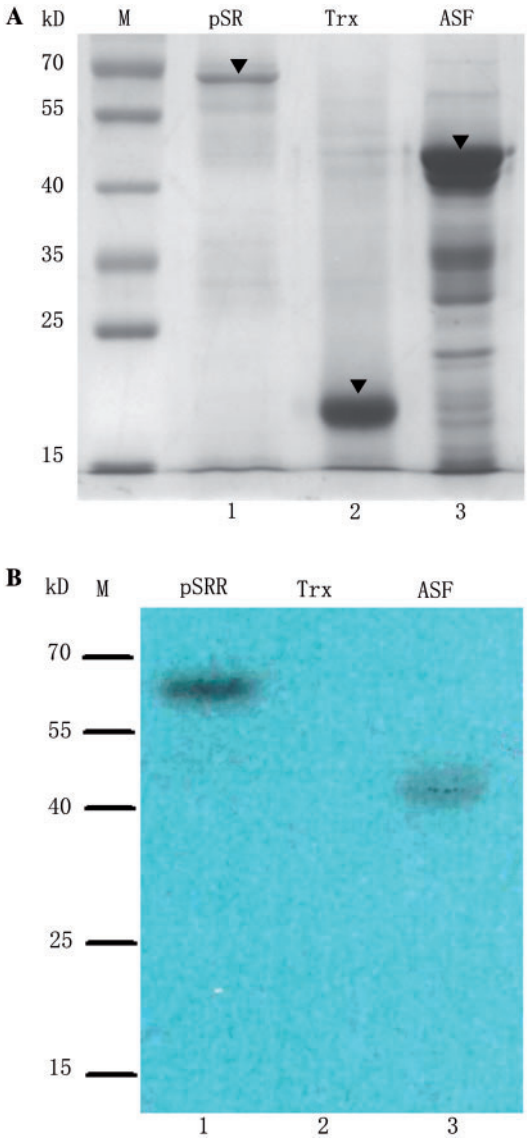

Fig. 3 Phosphorylation of PSR and ASF/SF2 by recombinant GST-PSRPK *in vitro*. (A) Bacterially expressed and purified recombinant proteins (1 μM) were incubated with 0.5 μM PSRPK in the presence of [γ-^{32}P] ATP at 30°C for 30 min. (B) The samples were then resolved by 10% SDS–PAGE and visualized by phosphoimaging. The positions of expressed PSR and ASF/SF2 are indicated by arrowheads.

with wt PSR. However, the results shown in Fig. 6B indicate that the mutation in His-PSR (dDock) protein positively affected catalysis by the SR protein kinase PSRPK. In addition, removal of a part of the docking motif (amino acids 424–434) significantly reduced the overall catalytic efficiency of the phosphorylation reaction (Fig. 6B, lane 3). Taken together, these results indicate that the docking-motif-like sequence was required for the phosphorylation of PSR by PSRPK *in vitro*.

Conserved domains of PSRPK are required for phosphorylation activity

In an earlier study, we showed that consensus motifs of PSRPK lie within two conserved fragments (amino acids 33–196 and 257–419) and that divergent motifs are distributed in the spacer region between the conserved N- and C-terminal domains. PROSITE software analysis revealed that PSRPK contains an ATP-binding region (ABR) ^{62}LGWGHFSTVWLA IDEKNGGREVALK^{86} and a serine/threonine protein kinases active-site signature (AS) ^{184}IIHTDLKPE NVLL^{196}, consistent with the signature of the corresponding peptide fragments of other SPRKs. To investigate whether these regions regulate the phosphorylationof PSR, we constructed deletion mutants of PSRPK lacking the ABR (dABR) and AS (dAS) (Fig. 7).

After expression and purification of dABR and dAS (Fig. 8A), the mutant proteins were each incubated with PSR in the presence of [γ-^{32}P] ATP. Mutations in both the ABR and AS drastically reduced the level of PSR phosphorylation compared with that of wt PSRPK (Fig. 8B, lanes 3 and 5). These findings imply that the ABR and AS play a major role in substrate phosphorylation.

Mutations at Lys^{86} and Asp^{188} negatively affect PSRPK phosphorylation activity

We next performed site-directed mutagenesis on two conserved residues Lys^{86} and Asp^{188} within ABR and AS, respectively, to further investigate their contribution to PSRPK phosphorylation activity. Lys at position 86 was replaced with Met (PSRPK mKM) and Asp at position 188 with Ala (PSRPK mDA).

The mutants were then incubated with purified, bacterially expressed PSR. After incubation in the presence of [γ-^{32}P] ATP, no phosphorylated bands were observed in the autoradiographs in comparison with wt PSRPK (Fig. 8B, lanes 3 and 5), indicating that mutation of these two residues abrogated the phosphorylation activity of PSRPK.

Discussion

We previously showed that PSRPK can phosphorylate ASF/SF2 and that the phosphorylation sites are located within the RS domain of the SR protein. In this study, we identified PSR as a novel member of the SR-related protein family in the true slime mold. We further demonstrated that PSR was phosphorylated by PSRPK *in vitro*, indicating that PSR is a physiological substrate for PSRPK in *P. polycephalum*.

PSR is suggested to be a novel member of the SR-related protein family since it contains one RS domain at the C-terminal end, which is a common feature of all members of the SR protein family, and a putative docking motif near the RS domain. The human SR protein ASF/SF2 contains two N-terminal RRMs followed by a short linker, connecting it to the C-terminal RS domain. The RS domain of ASF/SF2 is bifurcated in that it contains a tract of eight RS dipeptides (RS1 motif) followed by short interrupted stretches of RS dipeptides (RS2 motif). Moreover, the amino acid stretch between the RRMs and the RS domain (amino acids 183–197) containing the sequence ^{191}RVKVDGPR^{198} encompasses a docking motif on ASF/SF2 (*25*). However, important differences between

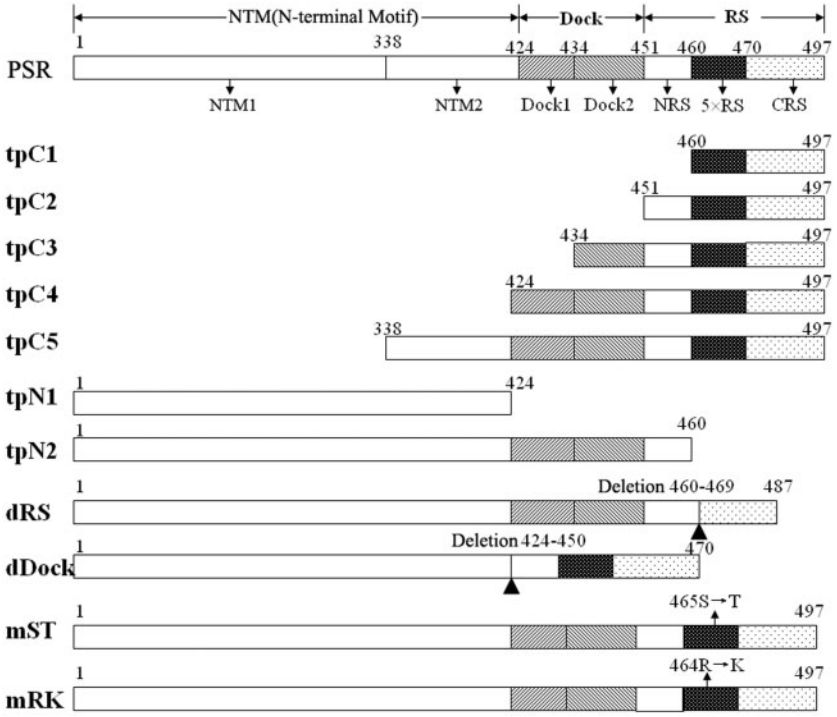

Fig. 4 Schematic representation of PSR, mutant PSR with the docking motif precisely deleted PSR(dDock), PSR (dRS) and a series of deletion constructs. The numbers corresponding to the beginning and end of the different domains are indicated.

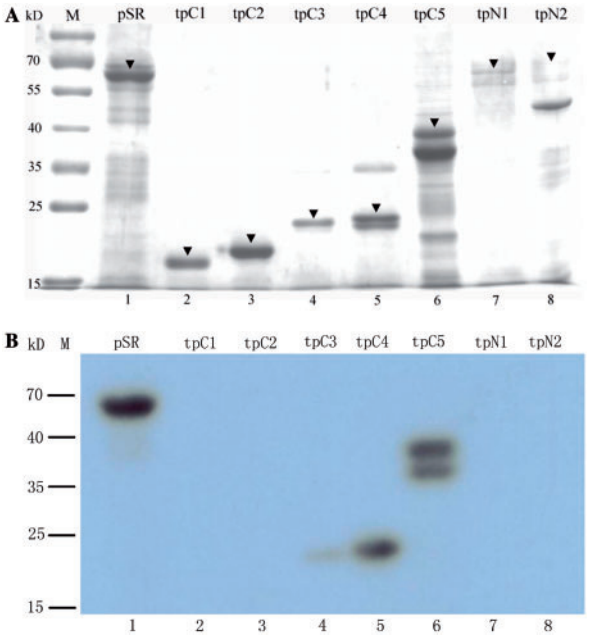

Fig. 5 PSR C-terminal contiguous RS dipeptides and the docking motif are sufficient for phosphorylation by PSRPK. (A) The indicated bacterially expressed Trx-fusion proteins indicated by arrowheads of equal amounts (1 μM) were incubated with PSRPK in the presence of [γ-^{32}P] ATP at 30°C for 30 min. (B) The samples were then resolved by 10% SDS–PAGE and visualized by phosphoimaging.

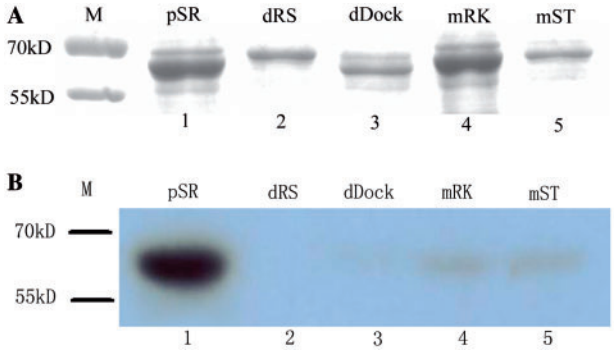

Fig. 6 PSR contiguous RS domain is the target of SR phosphorylation *in vitro*. (A) wt and mutated PSR were cloned downstream of a 6-His tag, expressed in bacteria, and then purified. (B) These were subjected to phosphorylation *in vitro* by recombinant PSRPK in the presence of [γ-^{32}P] ATP at 30°C for 30 min. The samples were then resolved by 10% SDS–PAGE and visualized by phosphoimaging.

the PSR of *P. polycephalum* and the metazoan SR protein ASF/SF2 were noted. First, the RS domain of PSR contains only one short stretch of basic amino acid, while ASF/SF2 contains a tract of eight contiguous RS dipeptides (RS1 motif) (*25*). The number of RS repeats is crucial for SR protein function, which depends on phosphorylation of the serines present (*26*). Second, an obvious RRM motif is absent in the *P. polycephalum* protein. To date, numerous RS-domain-containing proteins have been identified and these can be subdivided into two groups: (i) those that contain one or two RRMs in addition to the RS domain and (ii) those that do not contain an RRM (*27*). PSR belongs to the second group of RS domain proteins.

Our results demonstrate that as with other SR proteins, PSR can be phosphorylated by PSRPK *in vitro*, suggesting that PSR is an endogenous substrate for

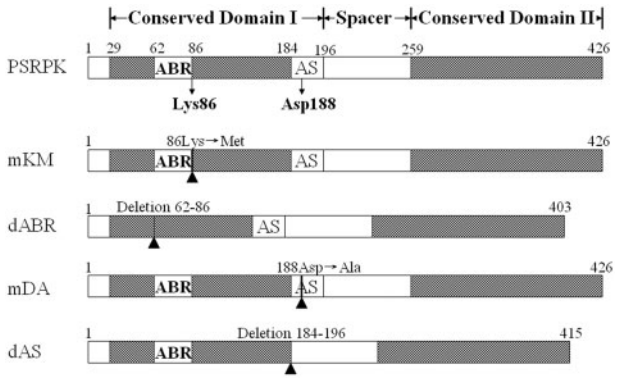

Fig. 7 Schematic representation of PSRPK and mutant PSRPKs with precisely deleted ABR (dAB), AS (dAS) and two other mutant constructs (mKM and mDA). The numbers corresponding to the beginning and end of different domains are indicated.

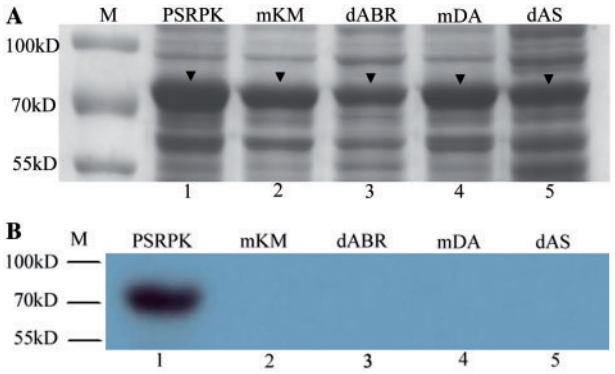

Fig. 8 Conserved domains and mutations on Lys86 and Asp188 of PSRPK are required for its phosphorylation activity. (A) PSRPK and mutated PSRPK were cloned downstream of a 6-His tag, expressed in bacteria and then purified. The positions of expressed PSRPK and mutated PSRPKs are indicated by arrowheads. (B) These proteins were tested *in vitro* for their abilities to phosphorylate recombinant PSR in the presence of [γ-^{32}P] ATP at 30°C for 30 min. The samples were then resolved by 10% SDS-PAGE and visualized by phosphoimaging.

PSRPK. In the previous study, we found that the RS domain of ASF/SF2 alone could be used as a phosphorylation substrate without the presence of sequences outside the SR domain (*20*). However, mutational analysis in the current study indicated that the truncated RS domains tpC1 (residues 460–497) and tpC2 (residues 451–497) of PSR could not be phosphorylated by PSRPK (Fig. 5B). Only the fragments containing both the docking motif and RS domains tpC3 (amino acids 434–497), tpC4 (amino acids 424–497) and tpC5 (amino acids 338–497) were phosphorylated by PSRPK (Fig. 5B). This indicates that the docking motif of PSR plays an important role in phosphorylation by PSRPK. Recent data suggest that during the phosphorylation of ASF/SF2 by SRPK1, the docking motif/docking groove interactions between ASF/SF2 and SRPK1 enable the kinase to lock onto the substrate and initiate phosphorylation at the center of the RS domain near the RS1/RS2 boundary of ASF/SF2, thus facilitating processive phosphorylation. The results suggest that additional serines in the RS domain are phosphorylated by SRPK1 once the docking motif of ASF/SF2 is deleted, indicating that the docking motif of ASF/SF2 limits its phosphorylation by SRPK1 (*16*, *25*). Remarkably, in yeast, removal of the docking groove in the kinase or the docking motif of the substrate does not reduce the overall catalytic efficiency of the phosphorylation reaction in any significant manner (*28*). However, our results suggest that the substrate significantly reduces the overall catalytic efficiency of phosphorylation by PSRPK once the docking motif of PSR is deleted (Fig. 6B). It is possible that the docking motif of PSR serves as the binding site for PSRPK and directs phosphorylation at another site.

The human alternative factor ASF/SF2 is activated by the multi-site phosphorylation of its C-terminal RS domain. The SRPK1 catalyses the selective phosphorylation of approximately a dozen serines in only the N-terminal portion of the RS domain (SR1). Arg^{214} was mutated to Lys in the contiguous RS dipeptides of SR1 domain and subsequent MALDI-TOF measurements indicated that the mutant was phosphorylated at 12 sites, suggesting that ASF/SF2 could tolerate a single Arg-to-Lys mutation in the RS1 domain without any effect on its phosphorylation activity (*16*). However, mutations of the RS dipeptide at arginines (Fig. 6B, mRK lane) or serines (Fig. 6B, mST lane) within the RS domain of PSR severely reduced its phosphorylation by PSRPK, indicating that the contiguous RS dipeptides near the C-terminus of PSR are necessary for phosphorylation by PSRPK. Previous data indicated that RRMs are not necessary for efficient phosphorylation of the RS domain but are important for maintaining processive phosphorylation of ASF/SF2 in the later phase of the reaction (*29*). Our results suggest that although PSR has no RRMs, its N-terminal Motif (NTM) is not necessary for efficient phosphorylation by PSRPK. Taken together, the data indicate that the SR domain and docking motif are sufficient for PSR phosphorylation. Further investigation is necessary to determine the mechanism by which PSR is phosphorylated by PSRPK.

In an earlier study, we showed that, similar to SRPK1, consensus motifs of PSRPK are found within two conserved fragments (amino acids 33–196 and amino acids 257–419) and the divergent motifs are divided into two halves by a large spacer sequence. Additionally, we found that PSRPK contains the ABR ^{62}LGWGHFSTVWLAIDEKNGGREVALK^{86} and AS ^{184}IIHTDLKPENVLL^{196} sequences, consistent with the signature of the corresponding peptide fragments of other SPRKs. Mutational analysis was performed on these functional domains of PSRPK in effort to understand their interaction with PSR. The dABR and dAS mutants obviously had reduced ability to phosphorylate PSR compared with the wt PSRPK (Fig. 8B), indicating that the ABR and AS played a major role in substrate phosphorylation.

Of the four amino acid residues studied here, mutations on Arg^{464} and Ser^{465} of PSR were found to severely reduced the ability of PSRPK to phosphorylate it.

As the contiguous RS dipeptides near the C-terminus are necessary for SR phosphorylation, Arg^{464} and Ser^{465} may represent two phosphorylation sites of PSR. Additionally, mutations of Lys^{86} and Asp^{188} of PSRPK likewise affected its phosphorylation activity. Combined with the tertiary structure of PSRPK visualized by the SWISS-MODEL software (*20*), our data led us to conclude that Lys^{86} and Asp^{188} are important sites for PSRPK activity on the ABR and AS, respectively.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of interest

None declared.

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