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PfSRPK1, a Novel Splicing-related Kinase from *Plasmodium falciparum*[*] [S]

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Even though it is increasingly evident that post-transcriptional events like mRNA processing and splicing may regulate gene expression and proteome diversity of malaria parasite *Plasmodium*, molecular mechanisms that regulate events like mRNA splicing in malaria parasite are poorly understood. Protein kinases control a wide variety of cellular events in almost all eukaryotes, including modulation of mRNA splicing, transport, and stability. We have identified a novel splicing-related protein kinase from *Plasmodium falciparum*, PfSRPK1. PfSRPK1 when incubated with parasite nuclear extracts inhibited RNA splicing, suggesting that it may control mRNA splicing in the parasite. PfSR1, a putative splicing factor from *P. falciparum*, was identified as a substrate of PfSRPK1. PfSR1 interacts with RNA and PfSRPK1 modulates its RNA binding. Early in the parasite development, PfSRPK1 and PfSR1 are present in the nucleus. These studies provide useful insights into the function of two potentially key components of *P. falciparum* mRNA splicing machinery.

Malaria is one of the most serious infectious diseases and causes several million deaths and clinical illness in hundreds of millions of people every year (1). One of the major problems of recent times has been the emergence of new drug-resistant strains of the malaria parasite *Plasmodium falciparum*. After invasion of the erythrocytes, the parasite can propagate asexually, giving rise to several new merozoites that invade fresh erythrocytes, which serve as hosts for the parasite to propagate. The blood stage infection is the cause of malaria pathogenesis. The parasite also undergoes sexual differentiation, which leads to the formation of male and female gametocytes. Upon ingestion of gametocytes by the *Anopheles* mosquito, further development continues inside the vector host. It is well known that signal transduction events mediated by protein kinases regulate diverse cellular processes. The importance of protein kinases in malaria parasite has been highlighted by a series of recent reports (2, 3), as these enzymes have been implicated in wide ranging events in both asexual and sexual stages of the parasite life cycle and, therefore, are considered as potential drug targets. Despite these reports, the information regarding signaling networks in the parasite is very limited.

Although it is clear that metazoan protein kinase cascades control gene expression by regulating transcriptional or post-transcriptional events, this area in *P. falciparum* biology is largely unexplored. *Plasmodium* displays a high degree of developmental control of gene expression (4). Strikingly, only a few transcription factors have been identified in the parasite leading to the speculation that post-transcriptional events may be pivotal in regulating gene expression in the parasite (5). Translational repression of some genes (6) and control of gene expression by antisense RNA are some of the post-transcriptional (7) events that have been implicated in regulating gene expression in the parasite. mRNA splicing is one of the major regulatory mechanisms that can modulate the expression of RNA transcripts and facilitate the synthesis of structurally and functionally distinct protein isoforms in most eukaryotes. Alternative mRNA splicing, which can cause proteome diversification from limited number of genes, has been observed for several *Plasmodium* genes that may affect the protein function (8–10). For instance, alternative splicing of MAEBL results in generation of different forms (9, 11). One of its isoforms, which has a transmembrane domain, is essential for invasion of mosquito salivary glands (11). The alternative splicing-mediated generation of protein isoforms can be one of the means via which the parasite may evade strategies devised to curb it. The knowledge of post-transcriptional mechanisms like mRNA splicing in *Plasmodium* is very limited (12, 13).

Typically, mRNA splicing occurs in the nucleus where the processed mRNA is stabilized and is exported to the cytoplasm for translation. Pre-mRNA splicing in mammalian cells is mediated by a multicomponent complex known as the spliceosome, which contains two classes of splicing factors; that is, small nuclear ribonucleoprotein particles (snRNPs) and non-snRNP splicing factors (14, 15). A series of interactions between pre-mRNA and small nuclear ribonucleoprotein particles during spliceosome assembly is critical for splice-site selection and, importantly, for establishing a catalytic core for the splicing reaction to occur in the spliceosome. Among the best characterized non-snRNP factors is the superfamily of arginine/serine-rich (RS)[5] domain-containing splicing factors

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[5] The abbreviations used are: RS domain containing splicing factor protein, RS domain, arginine-serine-rich domain; SR protein; MBP, myelin basic protein; SRPK, splicing-related protein kinase; KD, kinase dead; TSN, Tudor-SN.

(14). SR proteins are critical components of the spliceosome that influence both constitutive and alternative splicing of pre-mRNA. SR protein function is regulated by phosphorylation of their RS domains by multiple kinases, including a family of evolutionarily conserved SR protein-specific kinases (SRPKs). The SRPK family of kinases is unique as the kinases are capable of phosphorylating repetitive RS domains with remarkable specificity and efficiency (16, 17). In mammals and yeast, SRPKs have been implicated in several key functions like regulation of mRNA processing, nuclear import, germ line development, polyamine transport, and ion homeostasis (18–20). In addition to SRPKs, SR proteins are also phosphorylated by cdc28/cdc2-like kinases (Clk/Sty) (17). The cooperative phosphorylation of SR proteins is considered important for modulating its function and cellular localization (19). While cloning and identification of a Clk/Sty family member PfLAMMER has been reported (21), the regulation of SRPKs or Clk/Sty-like kinases is unexplored in *P. falciparum*.

We have identified and characterized a SRPK (PfSRPK1) and a splicing factor (PfSR1) in *P. falciparum*. PfSRPK1 phosphorylates PfSR1 and has the ability to modulate RNA splicing.

EXPERIMENTAL PROCEDURES

P. falciparum Cultures—*P. falciparum* 3D7 strain was cultured at 37 °C in RPMI 1640 medium using either AB+ human serum or 10% Albumax II (Invitrogen) as previously described (22). Synchronization of the parasites in culture was achieved by sorbitol treatment as reported earlier (23), and gametocytes were obtained as described previously (24).

In Silico Studies and Molecular Cloning of PfSRPK and PfSR Genes—A BLAST search was performed against the *P. falciparum* data base (PlasmoDB) using sequences for human SRPK1 (accession number NP_003128) and human SF2/ASF (accession number NP_008855). Two *P. falciparum* kinases (PFC0105w and PFl4_0408) that possess features of SRPKs emerged as orthologues of SRPKs and were named PfSRPK1 and PfSRPK2, respectively. PFE0160c, which exhibited homology to human SR protein SF2/ASF and possessed two RNA recognition motifs and an RS domain, was named as splicing factor PfSR1. The sequence information obtained for these genes from PlasmoDB was used to design PCR primers (supplemental Table S1). The kinase domain of PfSRPK1 and full-length PfSR1 was amplified by RT-PCR. Total RNA was isolated from asynchronous *P. falciparum* 3D7 cultures, and reverse transcription was performed using random hexamers provided in the Thermoscript reverse transcription-PCR kit (Invitrogen). Complimentary or genomic DNA was used as a template for PCR reactions, which were performed using platinum Taq polymerase (Invitrogen). Typically, after cycling parameters were used for these reactions: 94 °C for 2 min initial denaturation followed by 30 cycles at 94 °C for 30 s, 45 °C for 30 s, 68 °C for 2 min, and a final extension at 68 °C for 10 min. ΔPfSRPK1_K89M was generated by overlapping PCR mutagenesis using primer sets K89M_F and K89M_R (supplemental Table S1).

Expression and Purification of Recombinant Proteins—The kinase domain (KD) of PfSRPK1 is split in two segments (KDI and KDII), which are separated by a spacer region. The PfSRPK1 kinase domain without the spacer region, ΔPfSRPK1, was expressed as a recombinant protein. To achieve this, kinase domain I (KDI) and kinase domain II (KDII) of PfSRPK1 were amplified by RT-PCR using primers (PfSRPKF2/PfSRPKR2 and PfSRPKF3/PfSRPKR3) containing overhangs for SacI/HindIII and HindIII/XhoI restriction enzymes, respectively. The two PCR products were ligated and subsequently cloned in pET28 vector. Using this construct as a template, ΔPfSRPK1 was cloned in SmaI and NotI sites of pGEX-4T3 vector (Amersham Biosciences) to facilitate its expression as a GST fusion protein. PfSR1 was amplified using primers (PfSR1F1/PfSR1R1 and PfSR1F2/PfSR1R2) with overhangs for SacI/HindIII and BamHI/NotI for cloning in pET28 and pGEX4T3 vectors, respectively. Human ASF/SF2 was cloned in BamHI and XhoI sites in pGEX4T3 vector. *Escherichia coli* BL21-DE3 strain was transformed with pET28 plasmids, and BL21-RIL strain was used with pGEX4T3 plasmids. Bacterial cultures were grown in LB media containing either 25 μg/ml kanamycin or 100 μg/ml ampicillin and 25 μg/ml chloramphenicol. When cultures were in mid-logarithmic phase, expression of proteins was induced by 1 mM isopropyl β-D-1-thiogalactopyranoside at 18 °C for 14 h. Bacterial cells were harvested by centrifugation at 4000 × *g* for 30 min suspended in cold lysis buffer (50 mM Tris, pH 7.4, 2 mM EDTA, 1 mM dithiothreitol, 1% Triton X-100, and protease inhibitor mixture (Roche Applied Science), and sonication was performed for 6 cycles of 1 min each. The resulting cell debris was removed by centrifugation at 20,000 × *g* for 40 min at 4 °C. Fusion proteins from the cell lysates were affinity-purified using glutathione-Sepharose resin as described previously (22, 25). Briefly, the resin was washed with lysis buffer, and bound proteins were eluted with 50 mM Tris, pH 8.0, with 10 mM glutathione. Finally, purified proteins were dialyzed against 50 mM Tris, pH 7.4, 1 mM dithiothreitol, and 10% glycerol. Protein concentration was determined by densitometry analysis of Coomassie-stained gels. The recombinant His_6-PfSR1 protein was solubilized in extraction buffer (50 mM Tris, pH 7.4, 300 mM NaCl, and 8 M urea), and the clarified lysate was incubated with nickel nickel-nitrilotriacetic acid-agarose (Qiagen) with end-to-end shaking for 3 h at room temperature. The resin was poured into a column, washed with extraction buffer, and eluted with extraction buffer containing 0.5–10 mM imidazole. Fractions containing the protein of interest were pooled and dialyzed against buffers containing decreasing amounts of urea (25 mM Tris, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol, 0–6 M urea) to refold the recombinant protein. Recombinant proteins were estimated and analyzed by densitometry.

Immunoblotting, Immunofluorescence, and Immunoprecipitation—Polyclonal anti-PfSRPK1 serum was raised in rabbits using a synthetic peptide designed from the C-terminal sequence (NVNKINCKVINKKNS). This peptide was conjugated to keyhole limpet hemocyanin via an additional N-terminal cysteine residue (Peptron Inc.). Antiserum against PfSR1 was raised in mice using purified recombinant

GST-PfSR1 protein. Anti-GST antibodies were depleted by incubating the antisera with GST as described previously (26). For immunoblotting experiments, parasites were released from infected erythrocytes by 0.1% (w/v) saponin treatment. Cell-free protein extracts were prepared by suspending parasite pellets in a buffer containing 10 mM Tris, pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 1× Complete Protease inhibitor mixture (Roche Applied Science) using a syringe and a needle. Lysates were cleared by centrifugation at 14,000 × *g* for 30 min. After separation of lysate proteins on SDS-PAGE gels, proteins were transferred to a nitrocellulose membrane. Immunoblotting was performed using anti-PfSRPK1 and anti-PfSR1 antisera and horseradish peroxidase-labeled anti-rabbit IgG, and West Pico enhanced chemiluminescence substrate (Pierce) was used to develop the blots. PfSRPK1 was immunoprecipitated from parasite lysates using anti-SRPK1 antisera. For this purpose, erythrocyte-free parasite lysates were prepared as described above except phosphatase inhibitors (20 μM sodium fluoride, 20 μM-glycerophosphate, and 100 μM sodium vanadate) were added to the lysis buffer. 100 μg of lysate were incubated with the anti-sera at 4 °C overnight on an end-to-end shaker. Subsequently, antigen-antibody complexes were incubated with 50 μl of protein A-Sepharose beads for 2 h with end-to-end shaking. After washing with PBS several times, beads were finally resuspended in 50 μl of kinase assay buffer containing phosphatase inhibitors.

Immunofluorescence assays were performed on parasite smears fixed with methanol or acetone as described previously (27, 28). Briefly, smears were blocked in phosphate-buffered saline containing 0.05% saponin and 5% bovine serum albumin. Subsequently, incubation with primary antisera was performed for 2 h at room temperature or at 4 °C overnight followed by incubations with anti-mouse IgG labeled with Alexa 488 or Alexa 544 and 4′,6-diamidino-2-phenylindole (DAPI), which was used to stain parasite nucleus. The parasites were visualized using either a Zeiss Axio Imager fluorescence microscope, and images were processed using Adobe Photoshop or Axiovision software.

In Vitro Kinase Assays—Recombinant GST-ΔPfSRPK1 was assayed for catalytic activity in a buffer containing 50 mM Tris, pH 7.5, 10 mM magnesium chloride, 1 mM dithiothreitol, and 100 μM [γ-^{32}P]ATP (6000 Ci/mmol) using GST-PfSR1, His_6-PfSR1, and GST-ASF/SF2 proteins and RS peptide as phosphate-acceptor substrates. The RS peptide (RSPSYGRSRSRSRSRSRSRSNSRSRSY) was synthesized by Peptron Inc. Typically, reactions were carried out for 20–60 min at 30 °C and terminated by boiling the samples in SDS-PAGE sample buffer when proteins were used as phosphoacceptor substrates. Subsequently, reaction mixtures were electrophoresed on SDS-PAGE gel followed by autoradiography to visualize phosphate incorporation in the substrates. When RS peptide was used as the phosphor-acceptor substrate, reactions were stopped by spotting the samples on P11 phosphocellulose paper followed by washing the paper strips with 75 mM orthophosphoric acid. Phosphate incorporation was assessed by scintillation or Cerenkov counting of the P11 paper.

RNA Splicing Reactions—The 3′-end of PF13_0082 gene (cop-coated vesicle membrane protein p24 precursor, putative) consisting of exon 3 (174 bp), intron 3 (133 bp) and exon 4 (189 bp) was cloned in pGEMT easy, and the plasmid was linearized with NdeI. 1 μg of linearized plasmid DNA was used to generate radiolabeled RNA transcripts using RiboMax kit (Promega) and 50 μCi of [α-^{32}P]UTP (3000 Ci/mmol; PerkinElmer Life Sciences) following the manufacturer's instructions. Splicing of the purified RNA transcript was carried out in 50% v/v *P. falciparum* nuclear extracts, which were prepared by following a previously published protocol (29). Briefly, parasite pellets (obtained from 200 ml culture, 10% parasitemia) were incubated for 5 min in ice-cold lysis buffer (20 mM HEPES, pH 7.8, 10 mM KCl, 1 mM EDTA, and 1 mM DTT, 1 mM PMSF, 1% Nonidet P-40) and centrifuged at 2500 × *g* for 5 min at 4 °C. The pellet-containing nuclear fraction was washed twice with lysis buffer and centrifuged at 2500 × *g* for 5 min at 4 °C, resuspended in extraction buffer (20 mM HEPES, pH 7.8, 0.4 M NaCl, 1mM EDTA, 1 mM EGTA, 1 mM DTT, protease inhibitors), and incubated on ice for 15 min with vigorous shaking every 3 min. The lysate obtained after centrifugation was dialyzed against a buffer containing 20 mM HEPES, 0.2 mM EDTA, 50 mM KCl, 20% glycerol, 0.5 mM DTT, 2 mM PMSF). The *in vitro* transcribed 488-bp fragment of PF13_0082 pre-mRNA was incubated with intact or boiled nuclear extracts in the presence or absence of bacterially expressed ΔPfSRPK1 for 30 min at 25 °C (supplemental Fig. 6) in a reaction mix that contained 2 mM ATP, 2.5 mM $MgCl_2$, 3% PEG 8000, 0.6 M KH_2PO_4, RNasein nuclear extract 50% (v/v), radiolabeled RNA (40,000 cpm). On completion of splicing reactions, mixtures were treated with proteinase K (50 mg/ml), extracted with TRIzol, and precipitated with ethanol. RNA was fractionated by denaturing 5% PAGE, and splicing products were visualized by autoradiography (30).

UV Cross-linking of RNA and Protein—^{32}P-Labeled RNA transcript was prepared as described above. Recombinant proteins GST or GST-PfSR1 was incubated with labeled RNA in binding buffer (14 mM HEPES, pH 7.5, 6 mM Tris-Cl, pH 7.5, 1 mM EDTA, 1 mM DTT, and 60 mM KCl) for 30 min at 30 °C. The reaction mixture was transferred to an ice bath and irradiated with a short wavelength (254 nm) UV lamp (4 watt) held at a 3-cm distance from the reaction mixture for 30 min. After irradiation, RNase (2.5 units) was added, and the reaction mixture was incubated for 30 min at 37 °C to digest unbound RNA. The UV cross-linked products were boiled in Laemmli sample buffer for 3 min and analyzed on a discontinuous SDS–10% polyacrylamide gel (acrylamide/bisacrylamide ratio, 29:1). The gel was fixed with 7% acetic acid, and labeled complexes were visualized by autoradiography (31).

RESULTS

Identification and Molecular Cloning of Two SRPKs in P. falciparum—To identify SRPK orthologues in *P. falciparum*, human SRPK1 sequence was used to search the *P. falciparum* genome sequence *in silico*. Two PlasmoDB annotated gene products PFC0105w and PF14_0408 exhibited ~45 and ~25% sequence homology with the kinase domain of human SRPK1, respectively. PfSRPK1 has all the kinase subdomains

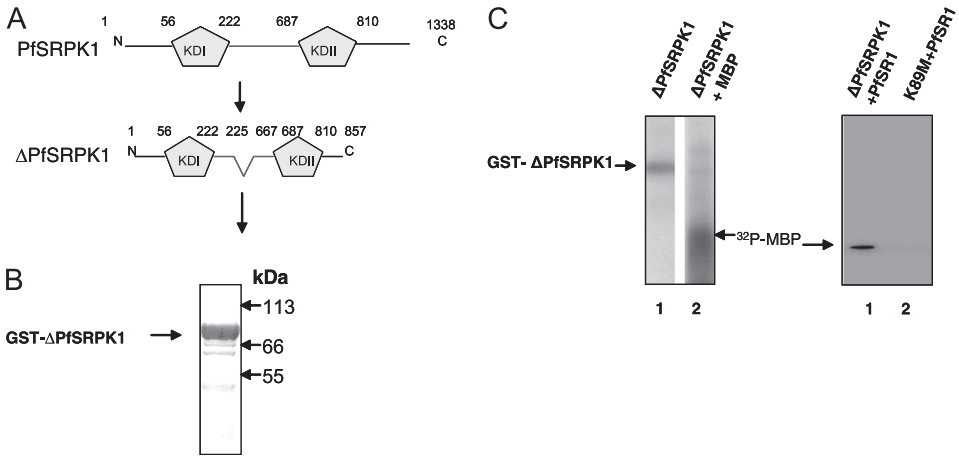

FIGURE 1. **PfSRPK1, a SRPK from *P. falciparum*.** *A* and *B*, shown is a schematic diagram illustrating the split kinase domain of PfSRPK1 along with the spacer region. The kinase domain lacking the spacer region, ΔPfSRPK1, was expressed as a GST fusion protein in *E. coli* (*B*). *C*, GST-ΔPfSRPK1 phosphorylates MBP. *Left panel*, GST-ΔPfSRPK1 was incubated with (*lane 2*) or without MBP (*lane 1*) in a kinase assay mix. *Right panel*, an equal amount of ΔPfSRPK1 (*lane 1*) or its K89M mutant (*lane 2*) was incubated with MBP in a kinase assay mix. Phosphate incorporation was monitored by autoradiography of the SDS-PAGE gel.

conserved and also possesses an insertion between domains VIb and VII, which is a signature for SRPKs (17) (supplemental Fig. S1). PfSRPK2 exhibited several differences when compared with PfSRPK1 and human SRPK1, most significantly the lack of glycines in ATP binding pocket (supplemental Fig. S1). These results agree with previous report on the kinome of *P. falciparum*, which predicted PFC0105w and PF14_0408 as members of SRPK family (2). The studies described here mainly deal with PfSRPK1 (PFC0105w). The uninterrupted kinase domain of PfSRPK1, obtained after the removal of the spacer region (Fig. 1*A*), was cloned in an expression vector to facilitate the expression of the recombinant ΔPfSRPK1 in *E. coli* (Fig. 1*B*). GST-ΔPfSRPK1 was able to phosphorylate myelin basic protein (MBP) *in vitro*, indicating that this spacer region-deleted version of the kinase was active (Fig. 1*C*) as was also the case with human SRPK1 (17). GST-ΔPfSRPK1 also exhibited autophosphorylation, which was reduced in the presence of MBP, most likely due to competition for the substrate binding site, thus suggesting that ΔPfSRPK1 may undergo trans-autophosphorylation. Most protein kinases possess a lysine near the ATP binding site, which is responsible for proper orientation of ATP and, therefore, is pivotal for catalysis. On comparison with other known protein kinases, Lys-89 emerged as a putative candidate for performing similar functions. The mutation of Lys-89 to Met resulted in loss of ΔPfSRPK1 activity (Fig. 1*C*, *right panel*), highlighting the importance of this residue in PfSRPK1 function.

PfSRPK1 Is Active in Constitutive Cis-splicing Reactions—To study the effect of PfSRPK1 in mRNA splicing, *in vitro* splicing reactions were performed using *P. falciparum* nuclear extracts as the source of splicing machinery. The pre-mRNA of cop-coated vesicle membrane protein p24 precursor (PF13_0082) gene was used as a candidate for splicing. This gene was chosen because it possesses conserved splicing-associated sequences at the exon-intron junctions; it has a GU at a 5′ splice site at the exon 3-intron 3 junction, adenosine at branch point, and AG at the 3′ splice site of intron 3-exon 4 junction (Fig. 2*A* and supplemental Fig. S3). The PlasmoDB-

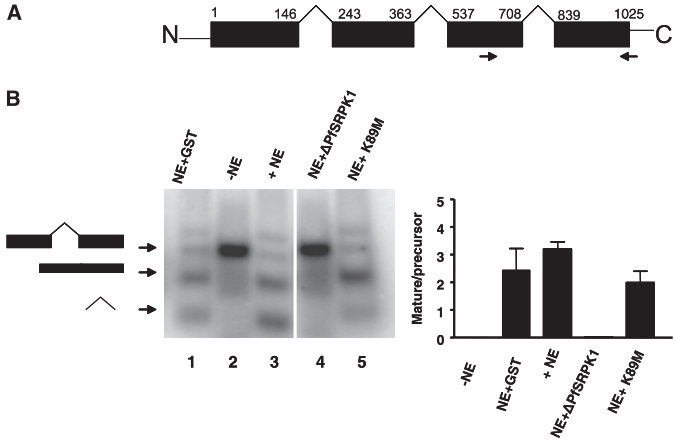

FIGURE 2. **PfΔSRPK1 modulates mRNA splicing.** *A*, shown is schematic representation of the PF13_0082 gene with PlasmoDB annotated exons/introns. *Arrows* indicate the region of the gene, which was used for *in vitro* transcription. *B*, the radiolabeled PF13_0082 pre-mRNA transcript corresponding to the region indicated in *panel A* (*lane 2*) was incubated with parasite nuclear extracts (*NE*) in the absence (*lane 3*) or presence of 100 ng of bacterially expressed ΔPfSRPK1 (*lane 4*) or K89M mutant (*lane 5*). Additional control experiments were performed in the presence of GST protein (*lane 1*). The reaction mix was separated by PAGE followed by phosphorimaging; various products were identified on the basis of their molecular size and are indicated in the figure. Results representative of more than three independent experiments are shown in the *left panel*. *Right panel*, an experiment similar to the one described in the left *panel* was performed, bands corresponding to the mature and precursor RNA were quantified by densitometry, and the ratio of their intensities was plotted. Mean of replicates from the experiment is shown; *error bars* represent the S.E.

annotated gene structure between exon 3 and 4 was confirmed by experimentally determining the cDNA and genomic DNA sequence. The predicted exon-intron boundary in this region matched with the experimentally determined sequence.[6] The 488-bp 3′-end region of this gene, which contains exons 3 and 4 that span an intron of 131 bp (Fig. 2*A*), was *in vitro* transcribed (supplemental Fig. S3). Incubation of this radiolabeled transcript with parasite nuclear extracts generated splicing fragments of the expected size (Fig. 2*B*, *lane 3*). When ΔPfSRPK1 was included in these assays, a significant

[6] P. K. Singh and P. Malhotra, unpublished results.

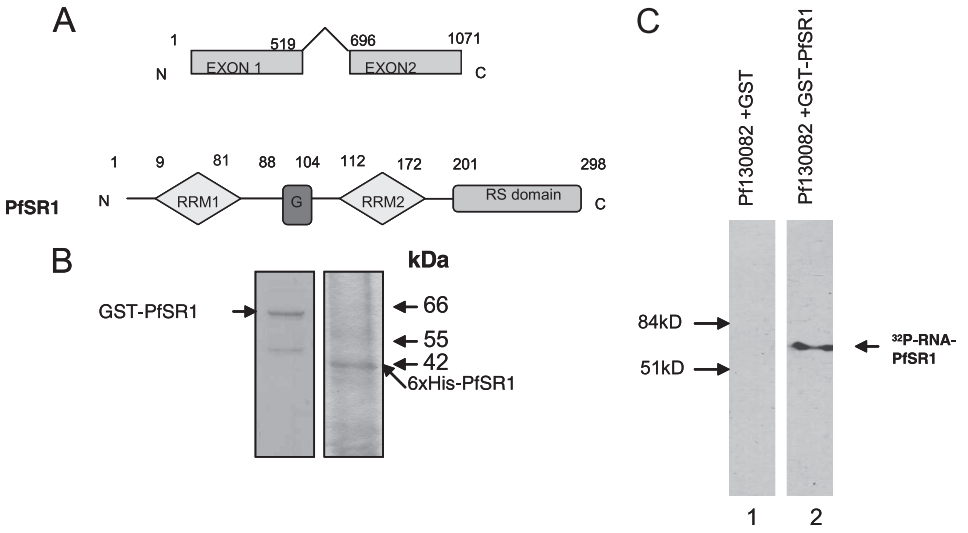

FIGURE 3. **PfSR1 is an SR protein that interacts with RNA.** *A*, shown is a schematic diagram illustrating the structure of PfSR1. *Top*, PfSR1 gene has a single intron that separates two exons. *Bottom*, PfSR1 comprises two RNA recognition motifs (RRM1 and RRM2) and an arginine-serine rich (RS) domain. *B*, PfSR1 was expressed as a GST fusion (*left panel*) or a His_6-tagged (*right panel*) protein in *E. coli* and was purified by affinity chromatography. *C*, GST (*lane 1*) or GST-PfSR1 (*lane 2*) was incubated with radiolabeled RNA transcript for PF13_0082 (see Fig. 2 and supplemental Fig. S3) followed by UV cross-linking. Subsequently, RNA bound protein was separated on a SDS-PAGE gel and detected by autoradiography.

inhibition in the formation of the spliced product was observed (Fig. 2*B*, *lane 4*). Control experiments performed with GST protein did not cause an inhibition in splicing (*lane 1*). Importantly, the K89M kinase dead mutant of ΔPfSRPK1 did not cause any significant changes to splicing (*lane 5*), suggesting that the observed effects were due to ΔPfSRPK1 catalytic activity. These studies demonstrate that PfSRPK1 may participate in mRNA splicing *in vitro*, and it has a negative effect on mRNA splicing mediated by the nuclear extract.

PfSR1, a SR-like Protein, Interacts with RNA—Having established that PfSRPK1 may modulate mRNA splicing, we probed its possible involvement with the components of the parasite splicing machinery. Typically, SRPKs from other organisms phosphorylate RS-rich domains in proteins, and often these domains are found in splicing factors SR proteins. We scanned the *Plasmodium* genome using RS domain of yeast and mammalian splicing factors. PFE0865c, which was named PfSR1, emerged as a candidate for mRNA splicing factor as it contained C-terminal RS domains and two RNA recognition motifs at the N terminus. PfSR1 shared ~54% sequence homology with human splicing factor 2 (ASF/SF2) (supplemental Fig. S2). PfSR1 cDNA was amplified by performing RT-PCR, and DNA sequencing revealed that the PfSR1 gene comprises 897 bp that includes a 177-bp intron (Fig. 3*A*). PfSR1 contains two RNA recognition motifs with conserved signature octapeptide (EFEDARDA) and heptapeptide (SWQDLKD) sequences (supplemental Fig. S2). A C-terminal domain with tandem repeats of arginines and serines (RS domain), which is typical of SR protein family members, is also present in PfSR1 (Fig. 3*A*, supplemental Fig. S2). The ability of PfSR1 to interact with RNA was tested by using recombinant GST-PfSR1 (Fig. 3*B*). For this purpose, *P. falciparum* gene PF13_0082 was selected (described above), and the radiolabeled RNA transcript corresponding to 450 bp segment of this gene was generated by *in vitro* transcription/translation in a cell free system (supplemental Fig. S3). This RNA transcript exhibited binding to PfSR1 in a UV cross-linking experiment (Fig. 3*C*).

PfSRPK1 Phosphorylates PfSR1 and Generates Phospho-epitopes That Are Different from Human SF2—Metabolic labeling experiments suggested that PfSR1 is phosphorylated in the parasite (data not shown). We tested if PfSRPK1 is the candidate kinase that may perform this function. Incubation of recombinant PfΔSRPK1 and not the K89M mutant with recombinant PfSR1 in a kinase assay resulted in phosphorylation of PfSR1 (Fig. 4*A*). A peptide corresponding to RS domain of SF2/ASF (RS-peptide, RSPSYGRSRSRSRSRSRSRSRSNSRSRSY), which was previously shown to be a substrate of human SRPK1 (32), was synthesized. ΔPfSRPK1 efficiently phosphorylated the RS peptide (Fig. 4*B*), suggesting that PfSRPK1 indeed has affinity for the RS-rich sequences. To further establish this, we expressed a deletion mutant of PfSR1, PfSR1ΔC, that lacked the last 128 amino acids that contain the RS domain. Although PfSR1 was phosphorylated by PfSRPK1, this mutant was not phosphorylated by the kinase, further suggesting that PfSRPK1 targets the RS domain of PfSR1 (Fig. 4*C*).

Previously, it was demonstrated that the hybridoma mAb104 recognizes phospho-epitopes generated as a result of phosphorylation of the RS domain of human SF2 by SRPK1 (33). Even though ΔPfSRPK1 phosphorylated PfSR1 (Fig, 4*D*, *right panel*, *lane 1*), mAb104 failed to recognize the phosphorylated form of PfSR1. Interestingly, ΔPfSRPK1 phosphorylated ASF/SF2 and was able to generate mAb104-positive phospho-epitopes on human SF2/ASF (Fig. 4*D*, *left panel*, *lane 1*). Close examination of the amino acid sequences of PfSR1 and SF2/ASF revealed that the distribution pattern and/or the number of RS repeats in the RS domain of PfSR1 is different from SF2 (supplemental Fig. S2). The variation in sequence and RS repeat pattern in two proteins may result in differential recognition by mAb104. It is also possible that the RS domains of the two proteins may adopt different confor-

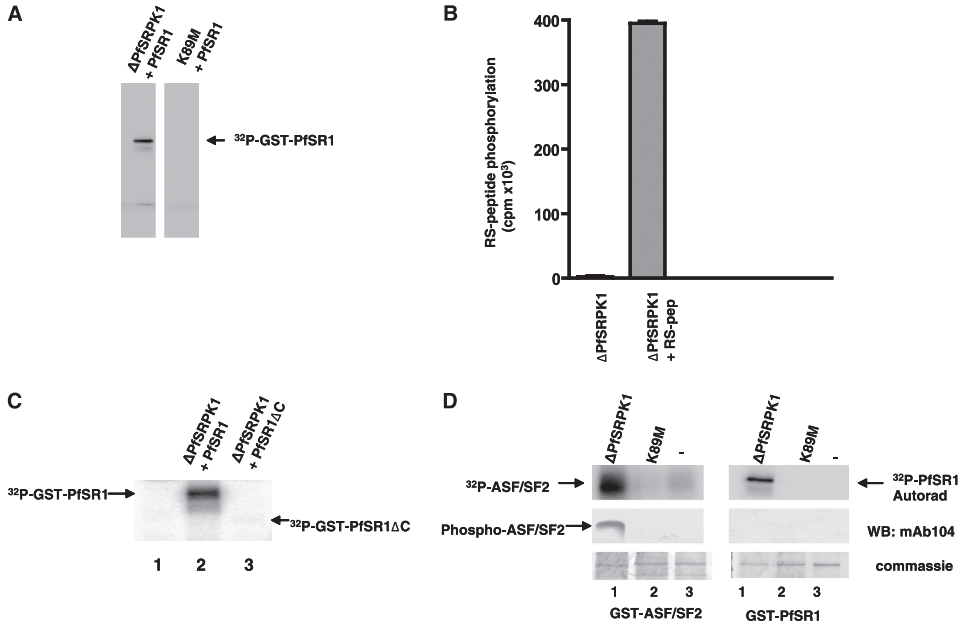

FIGURE 4. **PfSRPK1 phosphorylates PfSR1.** *A*, recombinant GST-ΔPfSRPK1 (*left panel*) or its kinase dead K89M mutant (*right panel*) was incubated with GST-PfSR1 (*A*) or RS peptide (*B*) in a kinase assay mix, and the reaction mixture was separated on a SDS-PAGE gel followed by autoradiography to detect the phosphorylation of PfSR1 (*A*). For assessing the phosphorylation of RS peptide (*B*), the reaction mix was spotted on a phosphocellulose paper followed by scintillation counting. Results representative of more than three independent experiments are shown in the figure. The mean of replicates from the experiment is shown; *error bars* represent the S.E. *C*, PfSR1ΔC, a C-terminal deletion mutant PfSR1 that lacked the last 128 amino acids of PfSR1 that contained the RS domain, was expressed. Equal amounts of this mutant (*lane 3*) or PfSR1 were (*lane 1*) were incubated in a kinase assay mix with ΔPfSRPK1 followed by SDS-PAGE and autoradiography. Unlike PfSR1, PfSR1ΔC was not phosphorylated by the kinase. *D*, equal amounts of GST-ASF/SF2 (*left panels*) or GST-PfSR1 (*right panels*) were incubated with GST-ΔPfSRPK1 (*lane 1*) or K89M (*lane 2*) in a kinase assay mix, and the phosphorylation reaction was carried out as mentioned above. The autoradiography of the SDS-PAGE gel revealed the labeling of phosphorylated proteins. Simultaneously, a similar phosphorylation experiment was also performed in which unlabeled ATP was used. In this case the reaction mix was used for Western blot (*WB*), which was performed using mAb104 (*middle panel*).

mations after phosphorylation, and mAb104 only recognizes the phosphorylated form of RS domain of SF2. It will be interesting to see if these variations account for differences in the mechanisms via which PfSR1 and SF2/ASF may operate in two different systems.

Association of PfSRPK1 and PfSR1—To evaluate if PfSRPK1 and PfSR1 associate in the parasite, co-immunoprecipitation experiments were performed. When PfSRPK1 was immunoprecipitated, PfSR1 was present in its immunoprecipitate (*IP*) (Fig. 5*A*). Recombinant GST-ΔPfSRPK1 and His_6-PfSR1 proteins were incubated *in vitro*, and GST pulldown assays were performed. The presence of His_6-PfSR1 in GST-ΔPfSRPK1-bound fractions indicated direct association of these proteins (Fig. 5*B*). GST by itself did not exhibit any significant binding to PfSR1. These observations established the interaction between PfSRPK1 with PfSR1 *in vitro* and in parasite extracts.

Given the association (Fig. 5, *A* and *B*) and phosphorylation of PfSR1 by PfSRPK1 (Fig. 4*A*), we next tested the effect of phosphorylation of PfSR1 on its RNA binding ability. To achieve this, ΔPfSRPK1-phosphorylated PfSR1 or its unphosphorylated form were incubated with the RNA transcript followed by cross- linking. The amount of RNA bound to the phosphorylated PfSR1 was significantly (~45%) reduced in comparison to its unphosphorylated form (Fig. 5*C*, *lane 3 versus lane 2*). These data may help explain PfSRPK1-mediated inhibition of splicing by the nuclear extracts; it is possible that the generation of negative charge due to phosphorylation of the PfSR1-RS domain may disrupt its binding to RNA, which may influence splicing.

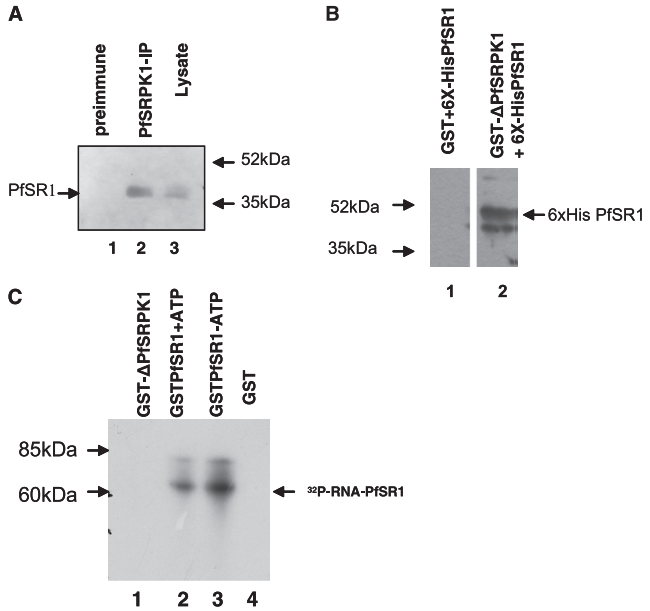

FIGURE 5. **PfSRPK1 interacts with PfSR1 and influences its interaction with RNA.** *A*, immunoprecipitation was performed using antisera against PfSRPK1 or pre-immune serum (control). Co-immunoprecipitation (*IP*) assays were electrophoresed on a SDS-PAGE gel followed by Western blotting with anti-PfSR1 antisera. *B*, equal amounts of His_6-PfSR1 were incubated with GST-ΔPfSRPK1 (*lane 2*) and GST (*lane 1*), and GST-pulldown assays were performed. PfSR1 associated with GST fusion protein was detected by Western blotting using anti-PfSR1 antisera. *C*, first, PfSR1 was phosphorylated by PfSRPK1 using unlabeled ATP in a kinase assay mix. *In vitro* transcribed and radiolabeled RNA transcript for PF13_0082 was incubated with either unphosphorylated PfSR1 (−*ATP*) or PfSRPK1-phosphorylated PfSR1 (+*ATP*) followed by UV-cross linking (as described for Fig. 2*C*) followed by autoradiography of SDS-PAGE gels. RNA-PfSR1 complex was observed at the expected molecular weight. Control experiment was performed using GST (*lane 4*) or GST-PfSRPK1 (*lane 1*).

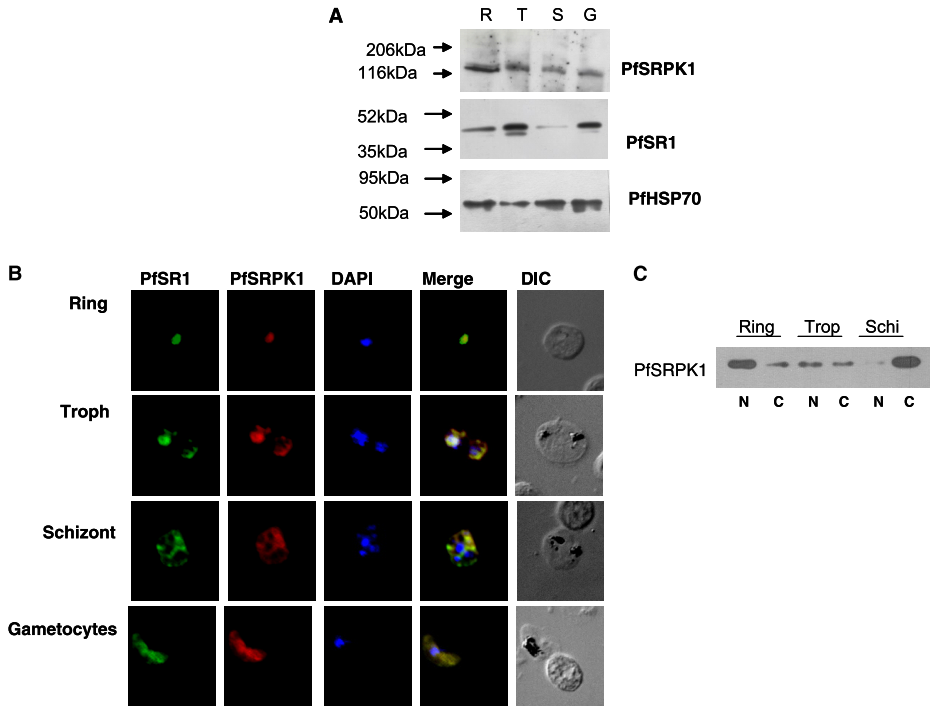

FIGURE 6. **Expression and localization of PfSRPK1 and PfSR1 during parasite development.** *A*, PfSRPK1 and PfSR1 are expressed in both asexual and gametocyte stages of *P. falciparum* life cycle. Equal amounts of cell lysate prepared from rings (*R* or *Ring*), trophozoites (*T* or *Trop*), schizonts (*S* or *Schi*), and gametocytes (*G*) were used for Western blot analysis, which was performed with anti-PfSR1 or anti-PfSRPK1 antisera. PfHSP70 was used as the loading control. *B*, immunofluorescence studies were performed on blood smears made from cultures containing parasites of different stages using antisera against PfSRPK1 (*red*), PfSR1 (*green*). Parasite nuclei were stained with DAPI. *DIC*, differential interference contrast. *C*, the cytoplasmic (*C*) and nuclear (*N*) fractions of parasite protein lysates prepared from different asexual parasitic stages were used for immunoblotting with PfSRPK1 antibody.

Localization of PfSRPK1 and PfSR1 during Parasite Development—To establish a connection between PfSRPK1 and PfSR1, their expression pattern in different developmental stages of the parasites was determined. To achieve this, antisera was raised against a peptide corresponding to a C-terminal region of PfSRPK1, which was absent from PfSRPK2. The Western blot analysis of protein lysates prepared from different blood stages of the parasite revealed a band of ~150 kDa (supplemental Fig. S5, Fig. 6*A*) that was close to the predicted size of PfSRPK1 protein. In addition, its expression was also detected in *P. falciparum* gametocytes (Fig. 6*A*), which was consistent with transcriptome data at PlasmoDB. Immunoblotting with anti-PfSR1 antisera identified a protein corresponding to the size of PfSR1. Like PfSRPK1, PfSR1 protein was also found in both asexual parasitic stages as well as gametocytes (Fig. 6*A*). Control pre-immune antisera from the same rabbit failed to detect this protein (data not shown).

To determine the cellular localization of PfSRPK1 and PfSR1, immunofluorescence assays were performed on parasites of different life-cycle stages. The staining for PfSR1 and PfSRPK1 overlapped in all parasitic stages (Fig. 6*B*). One of the striking finding of this study was the stage-specific nuclear trafficking of PfSRPK1 and PfSR1. These proteins were present predominantly inside the nucleus in the ring stages as indicated by co-localization with nuclear dye DAPI (Fig. 6*B*). PfSRPK1 and PfSR1 were present in the nucleus of ~75% of rings and young trophozoites that were analyzed. As the parasites matured, PfSRPK1 and PfSR1 seemed to be exported to nuclear periphery and/or cytoplasm in mature trophozoites. Both these proteins were predominantly in the cytoplasm of most (>80%) schizonts. Interestingly, both PfSRPK1 and PfSR1 are present in the cytoplasm of gametocytes with possibly only a small fraction in the nucleus (Fig. 6*B*). PfSR1 also appeared to be present on schizont-infected red blood corpuscles surface in some parasites (data not shown), although this needs further evaluation.

The nuclear-cytoplasmic localization was further confirmed by performing immunofluorescence assays with a nuclear protein PfTSN (supplemental Fig. S4) (34). PfSRPK1 co-localized with this nuclear protein in ring stages; both these proteins were in the nucleus. Although TSN continued to be present in the nucleus of trophozoites and schizonts, PfSRPK1 did not exhibit significant co-localization with it (supplemental Fig. S4). Furthermore, nuclear and cytoplasmic proteins were fractionated followed by immunoblotting. PfSRPK1 was predominantly in the nucleus of rings and in the cytoplasm of the mature parasites (Fig. 6*C*), which was in agreement with the immunofluorescence assays data.

DISCUSSION

Recent reports suggest that a large number of *Plasmodium* genes may undergo constitutive and alternative splicing (8). However, *P. falciparum* mRNA splicing machinery is still a "black box." In the case of higher eukaryotes, protein phosphorylation has been shown to play a role in the regulation of mRNA splicing (17, 35, 36). In the present study we have characterized PfSRPK1, an SR protein kinase in *P. falciparum*, and defined its role in interaction and localization of mRNA splicing factor PfSR1.

One of the features of SRPK and SR proteins is their dynamic nucleo-cytoplasmic shuttling, which is tightly regulated by phosphorylation (17, 18). It is known that the phosphorylation status of the RS domain of SR proteins is a key determining factor for their nuclear localization (37). In mammalian cells, SRPK is predominantly present in the cytoplasm, where it phosphorylates the RS domain of SF2/ASF, which causes its nuclear translocation (19, 37, 38). In the nucleus, protein kinase Clk/Sty further phosphorylates SF2/ASF ("hyperphosphorylated" form), which results in its release from nuclear speckles to the site of mRNA splicing. Subsequently, it is dephosphorylated by protein phosphatase PP1, resulting in its export to the cytoplasm (reviewed in Ref. 19). In comparison to SF2/ASF, SRPK1 spends only a short time in the nucleus, which is at G_2/M transition (39). We found that PfSRPK1 is predominantly inside the parasite nucleus during the early stages of development of asexual parasites. After mid-late trophozoite stages, there seems to be a switch in its localization to the cytoplasm. It is interesting to note that PfSR1 localization almost always goes "hand in hand" with that of PfSRPK1. Due to their interaction, it is possible that PfSRPK1 "piggybacks" PfSR1 in and out of the nucleus or vice versa. In the gametocyte, PfSRPK1 and PfSR1 were predominantly in the cytoplasm with some amounts in the nucleus. Given the function of SRPK and SR proteins in diverse events ranging from mRNA splicing in the nucleus (14) to translation repression in the cytoplasm (40), it will be interesting to determine whether these proteins participate in similar functions in the parasite. Translational repression of certain mRNA species has been observed in *Plasmodium* female gametocyte, which is achieved by storing the mRNA in the cytoplasm before fertilization (6). Given the presence of PfSR1 in gametocyte cytoplasm and its RNA binding ability, it will be worth investigating if PfSR1 and PfSRPK1 play a role in this process.

Recent reports suggest that the role and extent of mRNA splicing in *Plasmodium* may have been underplayed as a significant number of genes undergo splicing (8). To evaluate the function of PfSRPK1 in mRNA splicing, we selected gene PF13_0082. *P. falciparum* nuclear extracts were able to splice the pre-mRNA transcript for this gene and PfSRPK1, effectively blocked the splicing. It is well recognized that both hyper- and hypo-phosphorylation of SR proteins differentially effect mRNA splicing (19). SRPK1 and SR protein homologues have been identified in *Trypanosoma cruzi*. Although their direct involvement in the splicing of *T. cruzi* mRNA has not been demonstrated, TcSRPK1, when incubated with HeLa cell nuclear extracts, blocks the splicing of β-globin gene (36).

The phosphorylation of PfSR1 RS domain by PfSRPK1 inhibits its ability to interact with RNA. It is likely that the attenuation of RNA splicing by PfSRPK1 may be due to the decreased ability of phosphorylated PfSR1 to interact with the RNA transcript. Alternatively, hyperphosphorylation of PfSR1 by PfSRPK1 may disrupt its interaction with other members of the spliceosome assembly, which inhibits splicing by the parasite extract. Once the architecture of *Plasmodium* spliceosome and the identity of its components is clear, it will be interesting to study their interaction with PfSRPK1/PfSR1.

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PfSRPK1, a Novel Splicing-related Kinase from *Plasmodium falciparum*

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