

Phosphorylation of splicing factor SF1 on Ser20 by cGMP-dependent protein kinase regulates spliceosome assembly

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Splicing factor 1 (SF1) functions at early stages of pre-mRNA splicing and contributes to splice site recognition by interacting with the essential splicing factor U2AF65 and binding to the intron branch site. We have identified an 80 kDa substrate of cGMP-dependent protein kinase-I (PKG-I) isolated from rat brain, which is identical to SF1. PKG phosphorylates SF1 at Ser20, which inhibits the SF1-U2AF65 interaction leading to a block of pre-spliceosome assembly. Mutation of Ser20 to Ala or Thr also inhibits the interaction with U2AF65, indicating that Ser20 is essential for binding. SF1 is phosphorylated *in vitro* by PKG, but not by cAMP-dependent protein kinase A (PKA). Phosphorylation of SF1 also occurs in cultured neuronal cells and is increased on Ser20 in response to a cGMP analogue. These results suggest a new role for PKG in mammalian pre-mRNA splicing by regulating in a phosphorylation-dependent manner the association of SF1 with U2AF65 and spliceosome assembly.

Keywords: cyclic GMP/ pre-mRNA splicing/protein phosphorylation/U2AF

Introduction

The splicing of introns from nuclear pre-mRNA is catalysed by the spliceosome. This is a large ribonucleoprotein complex that assembles by a network of interactions between the pre-mRNA, small nuclear ribonucleoprotein particles (snRNPs) and many non-snRNP proteins (for a review see Krämer, 1996). These interactions contribute to the accuracy and specificity of splice site recognition that is necessary for the generation of mRNAs encoding functional proteins. Elements essential for the definition of splice sites are confined to the intron extremities and these are recognized several times during the assembly of the catalytically active spliceosome (Krämer, 1996; Reed, 1996). The 5' splice site is initially defined by base pairing with U1 snRNA in the early complex E, an interaction that is later replaced by U6 snRNA. In the same complex, two conserved elements that are located upstream of the 3' terminal AG dinucleotide of the intron, the branch site

and a polypyrimidine tract, are recognized by two protein factors. The large subunit of splicing factor U2AF (U2AF65) binds to the polypyrimidine tract, whereas SF1 (also termed branch point bridging protein, BBP) interacts with the branch site (Berglund *et al.*, 1997). U2AF65 and SF1 contact one another (Abovich and Rosbash, 1997; Rain *et al.*, 1998), resulting in cooperative binding of both proteins to the pre-mRNA (Berglund *et al.*, 1998a). Direct contacts between U2AF65 and the branch site have also been reported (Valcárcel *et al.*, 1996); however, it is not known whether binding of SF1 and U2AF65 to this region is mutually exclusive or occurs at the same time. SF1 is implicated in the bridging of splice sites by associating, directly or indirectly, with the U1 snRNP bound to the 5' splice site (Abovich and Rosbash, 1997; Bedford *et al.*, 1998). Members of the SR family of splicing factors, which contain Ser/Arg-rich domains, also participate in complex E formation and function in the juxtaposition of splice sites. These proteins can bind directly to the pre-mRNA and engage in interactions between a U1 snRNP-specific protein (U1 70K) and the 35 kDa subunit of U2AF (Fu, 1995). Binding of U2AF65 to the pre-mRNA and the presence of SF1 are essential for the following step in the assembly pathway, the formation of a short helix between U2 snRNA and the branch site, which results in assembly of pre-splicing complex A (Krämer, 1992; Zamore *et al.*, 1992). Completion of spliceosome assembly requires many additional proteins and involves the incorporation of the U4-U6/U5 snRNP into complex A, resulting in the formation of complex B. After a conformational change, complex C is formed and intron removal is catalysed within this complex in two consecutive transesterification reactions.

Splicing is regulated by reversible protein phosphorylation (see Manley and Tacke, 1996; Xiao and Manley, 1998). Among the 50 or more non-snRNP proteins that participate in splicing, the best studied targets for phosphorylation are the SR proteins. Their phosphorylation is required at the onset of spliceosome assembly and for the incorporation of the U4-U6/U5 snRNP into pre-splicing complex A, whereas dephosphorylation events are essential for catalysis. These modifications affect the interaction of SR proteins with each other, other splicing components and the pre-mRNA, thus contributing to the fine-tuning of the splicing reaction. Several kinases that phosphorylate SR proteins have been identified. Among these are SRPK1 and SRPK2, which specifically phosphorylate SR proteins (Gui *et al.*, 1994; Wang, H.Y. *et al.*, 1998) and the dual specificity kinase Clk/Sty (Colwill *et al.*, 1996). To date, the only non-SR protein shown to be phosphorylated is SF3b155/SAP155, a component of the U2 snRNP. SF3b155 is phosphorylated in spliceosomal complexes concomitant with or just after the first catalytic step (Wang, C. *et al.*, 1998) and is a target

for cyclin E/cyclin-dependent kinase 2 (Seghezzi *et al.*, 1998). In addition to regulating spliceosome assembly, phosphorylation affects the intranuclear distribution of splicing proteins (Misteli *et al.*, 1997 and references therein) and alternative splice site selection (Cardinali *et al.*, 1994; Duncan *et al.*, 1997; Kanopka *et al.*, 1998). Despite the advances in unravelling how the activities of splicing factors are modulated by phosphorylation, the upstream signals that trigger this regulation are largely unknown. Cellular signalling mechanisms that are, for example, mediated by insulin action (Chalfant *et al.*, 1995) or involve signalling by calcium and protein kinase C (PKC) (König *et al.*, 1998), can regulate alternative splicing events; however, in these cases the downstream targets remain to be explored.

A major signalling pathway common to many cells is mediated by the second messenger cGMP. Intracellular levels of cGMP are responsive to hormones of the atrial natriuretic peptide family and to a variety of other hormones which activate or induce the synthesis of nitric oxide synthase leading to the production of nitric oxide (Wang and Robinson, 1997). cGMP plays a role in a variety of physiological responses, from learning and memory to apoptosis. Major targets for cGMP signalling are the two Ser/Thr protein kinases PKG-I and PKG-II (Wang and Robinson, 1997 and references therein). Knockout mice lacking PKG-I have severe vascular and intestinal dysfunctions (Pfeifer *et al.*, 1998), while those lacking PKG-II have defects in intestinal secretion and severe dwarfism (Pfeifer *et al.*, 1996). PKG has a relatively limited substrate specificity compared with the highly related protein kinase A (PKA) and only a small number of substrates have been identified (Wang and Robinson, 1997). One target for PKG-I is c-raf1, phosphorylation of which blocks growth factor signalling to MAP kinase leading to reduced cellular proliferation (Sahasini *et al.*, 1998). New approaches have revealed novel putative PKG substrates in brain and other tissues by *in vitro* phosphorylation of proteins in tissue extracts (Wang and Robinson, 1995). The identification of these substrates is crucial for the understanding of PKG signalling. Here, we identify one of these substrates, an 80 kDa rat brain protein, as splicing factor SF1. PKG-I phosphorylates SF1 at Ser20, which interferes with the SF1-U2AF65 interaction and results in an inhibition of splicing complex formation. Our data suggest a new role for PKG and the cGMP signalling pathway in pre-mRNA processing.

Results

Identification of an 80 kDa brain PKG substrate as SF1

We have previously identified 48 proteins in rat brain that show a higher specificity for phosphorylation by PKG than by PKA (Wang and Robinson, 1995). An 80 kDa protein (P80) that was specifically phosphorylated by PKG was purified and characterized. A rat brain extract enriched in Ca²⁺-sensitive lipid-binding proteins was subjected to Q-Sepharose chromatography and proteins were eluted with a 0–400 mM NaCl gradient (Figure 1). Aliquots of each column fraction were phosphorylated in the presence of exogenous PKG or the catalytic subunit of PKA. Equal

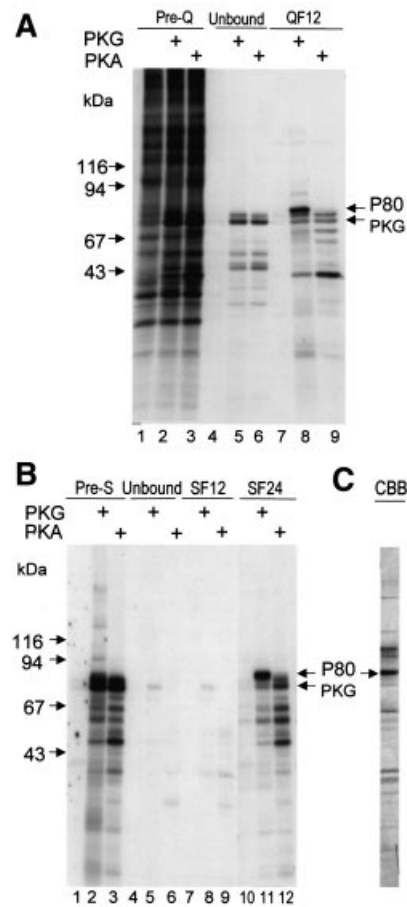


Fig. 1. Purification of an 80 kDa PKG substrate. (A) Rat brain extract enriched in Ca²⁺-sensitive lipid-binding proteins was applied to a Q-Sepharose column and proteins were eluted with a 0–400 mM NaCl gradient. Aliquots of the material applied to the column (lanes 1–3), the unbound fraction (lanes 4–6) and fraction 12 (lanes 7–9), were phosphorylated with [γ -³²P]ATP in the presence of PKG, cGMP and PKI, the catalytic subunit of PKA or no additions as indicated above the figure. Phosphoproteins were fractionated by SDS-PAGE on a 7.5–15% acrylamide gradient gel and visualized by autoradiography. The arrows on the right indicate autophosphorylated PKG (75 kDa) and P80. Molecular mass standards are shown on the left in kDa. (B) Q-Sepharose column fractions containing P80 were pooled, diluted, applied to an S-Sepharose column and eluted with a 0–400 mM NaCl gradient. Aliquots of the Q-Sepharose pool (lanes 1–3), the unbound material (lanes 4–6) and S-Sepharose fractions 12 (lanes 7–9) and 24 (lanes 10–12), were phosphorylated as above. (C) Coomassie Brilliant Blue (CBB) staining of proteins in S-Sepharose fraction 24 after concentration and desalting.

specific activities of PKG and PKA were used, as determined by phosphorylation of a synthetic peptide containing amino acids 8–21 of phospholamban (PL_{8–21}). As previously reported, virtually no specific PKG substrates could be detected in the crude cellular extract (Figure 1A, lanes 1–3) nor in the unbound material (lanes 4–6). P80 was eluted from the column at 100 mM NaCl (in fractions QF10–14) and was phosphorylated by PKG, but not PKA (Figure 1A, lanes 7–9). Fractions containing P80 were pooled and subjected to chromatography on S-Sepharose. Bound proteins were eluted with a 0–400 mM NaCl gradient and aliquots of each fraction were phosphorylated. P80 eluted at 200–250 mM NaCl (in fractions SF22–26; Figure 1B, lanes 10–12). At this point ~10 μ g of P80 were obtained that was sufficiently pure for amino acid sequencing (Figure 1C).

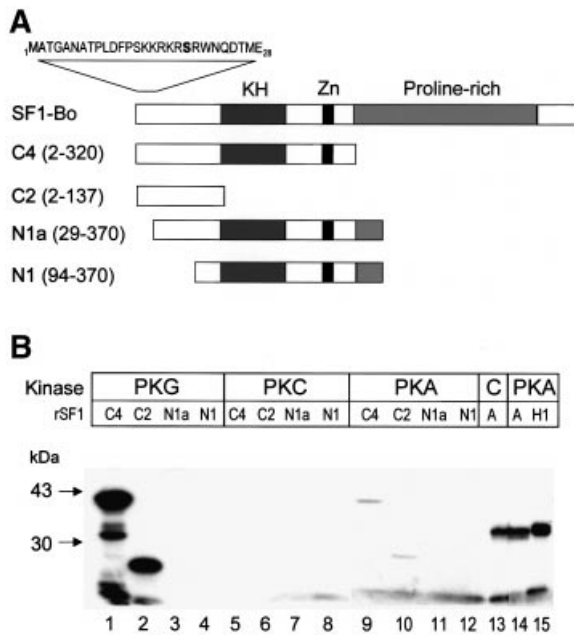


Fig. 2. The N-terminal 28 amino acids of recombinant SF1 are phosphorylated by PKG. (A) Schematic representation of full-length and mutant SF1 proteins. Key structural features are indicated by shading as follows: KH, KH domain; Zn, zinc knuckle; proline-rich region. The N-terminal 28 amino acids are shown on top, with Ser20 in bold text. Mutant SF1 proteins carry an N-terminal His₆-tag and contain the amino acids indicated. (B) Recombinant SF1 (rSF1) mutant proteins C4, C2, N1a and N1 (1 µg each) were phosphorylated in the presence of PKG (lanes 1–4), PKC (lanes 5–8) or PKA (lanes 9–12) as indicated above the figure. As controls, the phosphorylation of ASF/SF2 (A) with PKC (C, lane 13) and PKA (lane 14) and the phosphorylation of histone H1 with PKA (lane 15) are shown. Proteins were separated by SDS-PAGE on a 10% acrylamide gel and visualized by autoradiography. The migration of protein markers is indicated on the left in kDa.

P80 was in-gel digested with Endo-Lys-C and peptides were separated by high-performance liquid chromatography (HPLC). Amino acid sequencing of four of the major peptides revealed 100% identity of P80 with human SF1 (SF1_{19–28} RSRWNQDTME, SF1_{94–103} LNTREFR-TRK, SF1_{228–239} QGIETPEDQNDL and SF1_{298–308} PGDP-QSAQDKxR). The peptides originated from four distinct regions of the protein and single amino acid sequences were obtained in all cases, suggesting that P80 fully accounted for the Coomassie Blue-stained protein. To complete the identification of P80 as SF1, antibodies against two synthetic peptides of SF1 were raised in sheep and used for immunoblotting. The antibodies strongly reacted with recombinant SF1. P80 was detected in the crude brain extract and in S-Sepharose column fractions (data not shown). These results demonstrate that P80 is identical to SF1.

To study the relationship between P80 and SF1 further, recombinant His₆-tagged versions of SF1 (Figure 2A) were phosphorylated by PKG, PKA and PKC. SF1 proteins containing the N-terminal 320 (C4) or 137 (C2) amino acids were efficiently phosphorylated by PKG (Figure 2B, lanes 1 and 2). No or only weak phosphorylation was observed in the presence of PKC or the catalytic subunit of PKA, respectively (Figure 2B, lanes 5–12). The activity of PKC and PKA was confirmed by their ability to phosphorylate the SR protein ASF/SF2 or histone H1

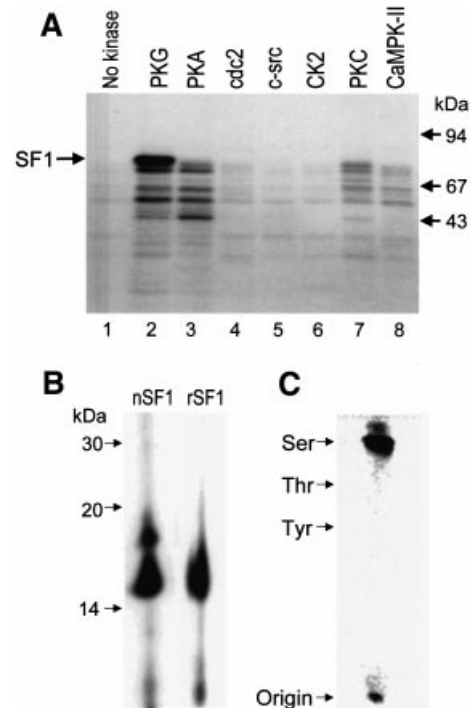


Fig. 3. Characterization of SF1 phosphorylation. (A) Specificity of SF1 phosphorylation. Purified rat brain SF1 was phosphorylated with [γ -³²P]ATP without any additions, in the presence of PKG, cGMP and PKI, the catalytic subunit of PKA, *cdc2*, *c-src*, CK2, PKC and CaMPK-II as indicated. Proteins were fractionated by SDS-PAGE on a 7.5–15% acrylamide gradient gel and phosphorylated proteins were visualized by autoradiography. The arrow on the left indicates SF1. Molecular mass standards are shown on the right in kDa. The results are representative of three experiments. (B) V8 protease phosphopeptide mapping. Purified rat brain native (nSF1) or recombinant SF1 (rSF1) were phosphorylated with [γ -³²P]ATP by PKG, fractionated by SDS-PAGE on a 7.5–15% acrylamide gradient gel and visualized by autoradiography. The phosphorylated proteins were excised from the dried gels using the autoradiograph as a template, digested with V8 protease, fractionated on a 15% gel and visualized by autoradiography. Molecular mass standards are shown on the left. (C) Phosphoamino acid analysis of SF1 purified from rat brain. SF1 was phosphorylated by PKG, separated by SDS-PAGE, excised from the dried gel and subjected to phosphoamino acid analysis. An autoradiograph is presented. The migration of phosphorylated serine, threonine or tyrosine standards is indicated on the left.

(Figure 2B, lanes 13–15), which are known substrates for these enzymes (Colwill *et al.*, 1996). When SF1 proteins with N-terminal truncations of 28 (N1a) or 93 (N1) amino acids were used, phosphorylation by PKG was abolished. Thus, recombinant SF1 serves as a specific substrate for PKG *in vitro* as observed with SF1 purified from rat brain. Moreover, the N-terminal 28 amino acids are essential for this reaction, suggesting that the phosphorylation site is localized within this region.

SF1 is specifically phosphorylated by PKG but not by other protein kinases

Next the specificity of SF1 phosphorylation by various protein kinases was addressed. As shown in Figure 3A, rat brain SF1 was an excellent substrate for PKG, but was not phosphorylated by six other protein kinases: the catalytic subunit of PKA, *cdc2* kinase, *c-src*, casein kinase 2 (CK2), PKC and calcium and calmodulin-dependent protein kinase II (CaMPK-II). The activity of each kinase

was confirmed in separate experiments by their ability to phosphorylate purified rat brain dynamin I (PKC, *cdc2* and CK2) or to phosphorylate proteins present in other S-Sepharose column fractions (data not shown). This result demonstrates that SF1 is a specific substrate for PKG, but not for six other protein kinases tested. As shown in Figure 2B, recombinant SF1-C4 is also preferentially phosphorylated by PKG compared with PKA, and is not phosphorylated by PKC.

PKG phosphorylates SF1 at Ser20 *in vitro*

To determine whether recombinant and purified SF1 are phosphorylated by PKG at the same site(s), native SF1 purified from rat brain (nSF1) and recombinant SF1-C4 (rSF1) were phosphorylated with PKG and subjected to phosphopeptide mapping with V8 protease (Figure 3B). A major phosphorylated peptide of 16 kDa was detected with both proteins, suggesting that PKG phosphorylates the same site or region in native and recombinant SF1. The minor, slower migrating phosphopeptide detected with nSF1 represents a partial proteolytic product that in some experiments is further digested to the 16 kDa peptide. PKG is a Ser/Thr-specific protein kinase and phosphoamino acid analysis revealed that PKG phosphorylated nSF1 exclusively on Ser (Figure 3C).

Studies with synthetic peptide libraries (Tegge *et al.*, 1995) and comparison of the sequences of the few known PKG substrates (Wang and Robinson, 1997) have revealed the motif RKxS/T (x being any amino acid) as the optimal sequence context for PKG phosphorylation. Amino acids 17–20 of SF1 showed an excellent match to this motif (Figure 2A). Taken together with the observation that the N-terminal 28 amino acids of SF1 were required for phosphorylation, Ser20 would be expected to represent the phosphate acceptor. To test this, PKG-phosphorylated SF1-C4 was subjected to digestion by Lysyl-endopeptidase C and the peptides were separated by HPLC. Two peaks of radioactivity were obtained. Since SF1 was exclusively phosphorylated on Ser, both fractions were derivatized to convert phosphoserine to S-propylcysteine, which is detectable by HPLC because of its specific elution after leucine (data not shown). The sequences of both peptides contained Ser20 (RSRWNQDTME and RKRSRWNQDT) and the derived moiety revealed that the phosphorylation site was Ser20.

To confirm this result, Ser20 of SF1-C4 was mutated to Thr (SF1-C4-S20T) or Ala (SF1-C4-S20A). Compared with SF1-C4, SF1-C4-S20T was phosphorylated by PKG at a greatly reduced rate (Figure 4). Thus, Ser serves as a much better target for PKG than Thr in the context of this sequence. Mutation of Ser20 to Ala completely abolished phosphorylation, providing further evidence that Ser20 represents the only PKG phosphorylation site in SF1.

The kinetics of phosphorylation were determined with a synthetic peptide, SF1_{15–25}, that included Ser20. The kinetics (K_m and V_{max}) of phosphorylation of SF1_{15–25} by PKG and PKA were compared with those of one of the best PKG peptide substrates, PL_{8–21}, which exhibits similar kinetics for PKG and PKA (Kemp and Pearson, 1991; Table I). SF1_{15–25} served as a high affinity PKG substrate and was phosphorylated at similar rates as PL_{8–21} (Table I). In contrast to PL_{8–21}, however, SF1_{15–25} was phosphorylated 13.6-fold less by PKA. Therefore the specificity

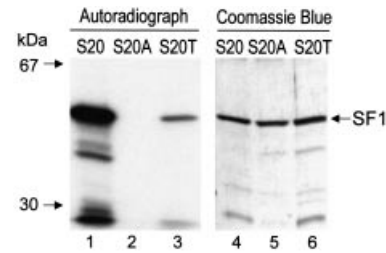


Fig. 4. Mutation of Ser20 to Thr or Ala inhibits PKG phosphorylation of SF1. SF1-C4 proteins carrying the wild-type Ser20 (lanes 1 and 4) and mutant proteins SF1-C4-S20A (lanes 2 and 5) or SF1-C4-S20T (lanes 3 and 6), were phosphorylated in the presence of PKG, cGMP and [γ - 32 P]ATP. Proteins were separated by SDS-PAGE in a 12% acrylamide gel, stained with Coomassie Blue (lanes 4–6) and phosphorylated proteins were visualized by autoradiography (lanes 1–3).

for phosphorylation by PKG as compared with PKA was the same for SF1_{15–25} and the native and recombinant proteins.

Phosphorylation of SF1 on Ser20 in intact cells

To determine whether SF1 phosphorylation could be of physiological relevance, the phosphorylation of SF1 in intact cells was investigated using the rat brain-derived neuronal-like cell line B35 (Schubert *et al.*, 1974). These cells differentiate in response to cAMP stimulation and extend axonal-like processes. Differentiated cells were labelled with 32 P_i, washed and stimulated with membrane-permeable cGMP or cAMP analogues (8-pCPT-cGMP or 8-pCPT-cAMP). SF1 was immunoprecipitated with the anti-SF1 peptide antibody and immunoprecipitates were separated by SDS-PAGE in parallel with *in vitro* PKG-phosphorylated SF1 as a marker. A protein co-migrating with SF1 was weakly phosphorylated in B35 cells and increased in phosphorylation in response to stimulation with the cGMP analogue (Figure 5, lanes 4 and 5). The response was specific, since the cAMP analogue did not induce phosphorylation of the protein (Figure 5, lane 6). Control experiments indicated that SF1 was not immunoprecipitated with pre-immune serum (Figure 5, lanes 7–9). A faster migrating 76 kDa 32 P-labelled band immunoprecipitated with the SF1 serum was non-specific as it was also observed with pre-immune serum. This result suggests that SF1 is phosphorylated *in vivo* in response to extracellular stimuli that activate the cGMP signalling pathway.

The next aim was to determine whether SF1 was phosphorylated on Ser20 *in vivo*. Polyclonal antibodies were raised in sheep against a synthetic peptide encompassing the phosphorylation site and which included a phosphorylated serine [SF1_{17–24} RKRS(P)RWNQ]. The specificity of the antibodies was confirmed by Western blotting. Anti-SF1 antibodies recognized recombinant SF1, regardless of its phosphorylation by PKG (Figure 6A), while the anti-phospho-Ser20 antibodies preferentially detected PKG-phosphorylated SF1 (Figure 6B). When SF1 was immunoprecipitated from duplicate plates of B35 cells with protein G-Sepharose-purified anti-SF1 antibodies, a constant amount of SF1 was recovered from control and cGMP-stimulated cells. This was determined by probing the blots with the same anti-SF1 antibodies as those used for immunoprecipitation (Figure 6C). Notably,

Table I. Kinetics of phosphorylation of synthetic SF1 and PL peptide substrates by PKG and PKA

Substrate	K_m (μ M)		V_{max} (μ mol/mg/min)		V_{max}/K_m		Specificity index (PKG/PKA)
	PKG	PKA	PKG	PKA	PKG	PKA	
SF1 ₁₅₋₂₅	29.4 \pm 3.7	51.8 \pm 9.5	6.41 \pm 0.4	0.83 \pm 0.1	0.22	0.016	13.6
PL ₈₋₂₁	37.4 \pm 2.8	18.3 \pm 2.8	6.0 \pm 0.2	4.1 \pm 0.5	0.16	0.22	0.73

The synthetic peptides SF1₁₅₋₂₅ and PL₈₋₂₁ were used as substrates in kinase assays. The apparent kinetic constants were determined from Lineweaver–Burke plots with the programme Enzfit (mean \pm SD for $n = 3$). The substrate specificity was determined by calculating the V_{max}/K_m ratio for each kinase (a higher ratio indicates a better substrate). The specificity index is the ratio of V_{max}/K_m for PKG divided by the V_{max}/K_m for PKA (a higher value indicates the peptide is a better substrate for PKG relative to PKA).

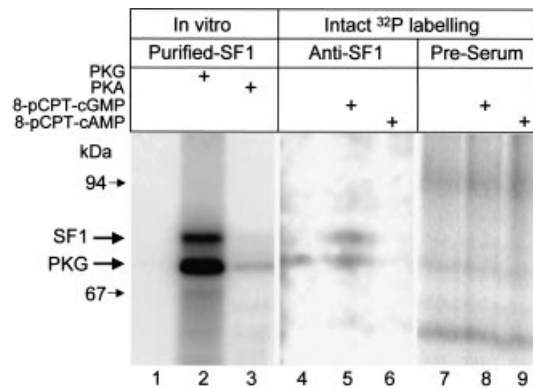


Fig. 5. Phosphorylation of SF1 is increased by cGMP analogues *in vivo*. B35 rat brain neuron-derived cells were differentiated for 2 days with dibutyl cAMP, washed and labelled with 32 P_i. Cells were then washed and stimulated for 30 min with the cGMP or cAMP analogues indicated. Cells were lysed and proteins were immunoprecipitated with a sheep anti-SF1 peptide antibody (lanes 4–6) or pre-immune serum (lanes 7–9). The immunoprecipitates were separated on a 7.5–15% acrylamide gel and phosphoproteins were visualized by autoradiography. In parallel lanes of the same gel, PKG-phosphorylated SF1 was run as a standard for molecular mass comparison (lanes 1–3, see experimental details in Figure 1A). Results are representative of three independent experiments, performed in duplicate.

the non-specific 76 kDa band (Figure 5) was not detected. The blot was then stripped and re-probed with protein G–Sepharose-purified anti-phospho-Ser20 antibodies, which primarily detected SF1 from the cells that had been stimulated with the cGMP analogue (Figure 6D). This demonstrates that SF1 is phosphorylated *in vivo* on Ser20 in response to a cGMP stimulus.

Phosphorylation of SF1 regulates its interaction with U2AF65 and inhibits pre-spliceosome assembly

SF1 functions during early stages of spliceosome assembly by binding to the intron branch site and interacting with splicing factor U2AF65 (see Introduction). To determine whether these activities are modulated by phosphorylation we first examined the interaction of phosphorylated SF1 with U2AF65. His₆-tagged SF1-C4 was phosphorylated or mock-phosphorylated (no ATP) by PKG, purified by metal-affinity chromatography and incubated with glutathione *S*-transferase (GST)-tagged U2AF65 bound to glutathione–agarose beads. After removal of unbound material, bound proteins were eluted with SDS sample buffer, separated by SDS–PAGE and blotted to a nylon membrane. SF1-C4 was detected by antibodies directed against the N-terminal His₆-tag. As previously shown

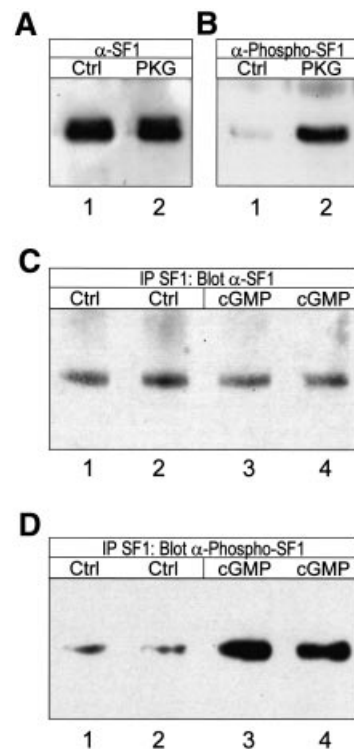


Fig. 6. PKG phosphorylates Ser20 *in vivo*. (A and B) Phosphorylation site-specific antibodies. SF1-C4 (100 ng) was mock-phosphorylated (lane 1) or phosphorylated with PKG (lane 2), run on a 10% acrylamide gel and transferred to nitrocellulose. The blot was probed with antibodies to SF1 [α -SF1, (A)] or to phospho-Ser20 SF1 [α -phospho-SF1, (B)]. Results are representative of six experiments. (C and D) Phosphorylation of Ser20 in B35 cells. Duplicate plates of differentiated B35 cells were stimulated with medium (lanes 1 and 2) or medium with 8-pCPT-cGMP (cGMP, lanes 3 and 4) as described in the legend to Figure 5. SF1 was enriched from cell lysates by batch elution from Q-Sepharose resin, immunoprecipitated with the anti-SF1 antibodies, run on a gel and transferred to nitrocellulose. The membrane was initially probed with α -SF1 (C) to show constant protein recovery. After stripping, the membrane was re-probed with α -phospho-SF1 to detect Ser20 phosphorylation (D). Results are from an experiment performed in duplicate and similar results were obtained in two further experiments omitting the Q-Sepharose step.

for non-phosphorylated SF1 (Rain *et al.*, 1998), mock-phosphorylated SF1-C4 bound to GST–U2AF65 (Figure 7A). After phosphorylation, the SF1–U2AF65 interaction was severely reduced at all concentrations of SF1-C4 tested. No binding was observed with GST bound to the beads or with glutathione beads alone. As a control, a similar experiment was performed after incubation of SF1-C4 with PKA, which results in only weak phosphorylation of the protein. In this case the mock-

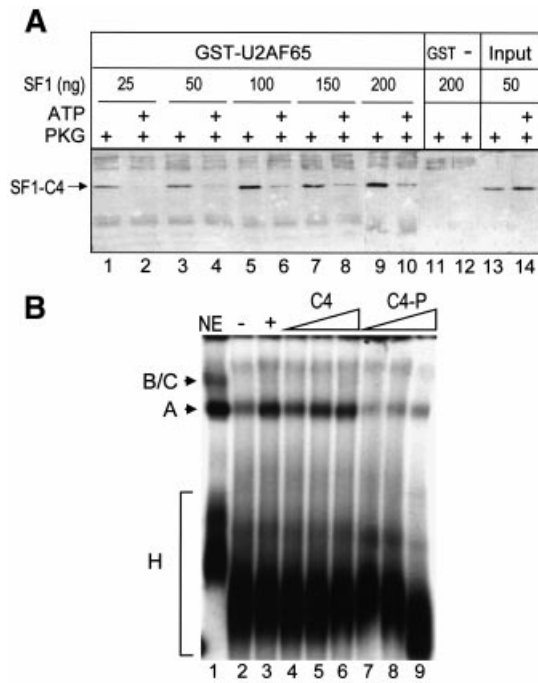


Fig. 7. PKG phosphorylation of Ser20 in SF1 inhibits its interaction with U2AF65 and pre-spliceosome assembly. **(A)** Effect of PKG phosphorylation on the SF1–U2AF65 interaction. Increasing amounts of SF1–C4 were incubated with PKG in the presence of 1 mM ATP or were mock-phosphorylated in the absence of ATP as indicated above the figure. Proteins were added to glutathione beads coated with GST–U2AF65 (lanes 1–10), GST–glutathione beads (lane 11) or to glutathione beads alone (lane 12). After washing, bound proteins were eluted, separated on a 12% acrylamide gel, blotted onto a nylon membrane and visualized after incubation with anti-poly-His antibodies. Input proteins corresponding to 50 ng of mock-phosphorylated or phosphorylated SF1–C4 are shown in lanes 13 and 14, respectively. **(B)** Effect of PKG phosphorylation on pre-spliceosome assembly. Splicing complexes were assembled on a 32 P-labelled pre-mRNA substrate in the presence of HeLa cell nuclear extract (NE, lane 1) or partially purified splicing components in the presence of 1 μ M okadaic acid (lanes 2–9). The reactions contained the following additions: buffer (lane 2), SF1 isolated from HeLa cells (lane 3), 3, 8 and 14 ng of SF1–C4 control (lanes 4–6) or phosphorylated SF1–C4 (lanes 7–9) in a 10 μ l reaction. Splicing complexes were separated in a non-denaturing 4% polyacrylamide gel and visualized by autoradiography. The migration of complexes H, A and B/C is indicated on the left. The background activity seen in lanes 2 and 7–9 is due to a contamination of the SF3a/b fraction with SF1.

phosphorylated and phosphorylated proteins interacted with U2AF65 with no apparent differences (data not shown). We conclude that the specific phosphorylation of SF1 at Ser20 by PKG inhibits its interaction with U2AF65.

Analysis of functional domains in SF1 has revealed that sequences encompassing the KH domain (amino acids 136–228) are essential for RNA binding (Berglund *et al.*, 1998b; Rain *et al.*, 1998). Consistent with these results, phosphorylation of SF1–C4 did not affect the ability of the protein to bind RNA, as tested by UV crosslinking of the protein to a pre-mRNA derived from the adenovirus major late transcription unit (data not shown).

The N-terminal 28 amino acids of SF1 are essential for both binding to U2AF65 and pre-spliceosome formation (Rain *et al.*, 1998). Given the result that SF1 phosphorylated at Ser20 cannot bind to U2AF65 we anticipated that PKG phosphorylation would also inhibit the activity of

SF1 in spliceosome assembly. SF1–C4 was PKG-phosphorylated, purified and added to reactions containing partially purified splicing components that are essential for the formation of pre-splicing complex A (Figure 7B; Krämer and Utans, 1991). Splicing complexes A and B/C are assembled in HeLa cell nuclear extracts (Figure 7B, lane 1). Complex A was efficiently assembled in the reconstituted system when SF1 purified from HeLa cells was added (Figure 7B, lane 3), as compared with a control performed in the absence of SF1 (lane 2; the background activity observed in this reaction is due to a small contamination of the SF3a/b fraction with SF1). Complex A was also formed upon addition of increasing amounts of mock-phosphorylated SF1–C4 (Figure 7B, lanes 4–6). In contrast, PKG phosphorylation of SF1–C4 completely inhibited spliceosome assembly (Figure 7B, lanes 7–9). Incubations were performed in the presence of okadaic acid to inhibit protein phosphatases present in fractions containing the partially purified splicing factors. In the absence of okadaic acid the phosphorylated SF1–C4 was dephosphorylated within 5 min of incubation at 30°C and was fully active in complex assembly (data not shown). These results demonstrate that phosphorylation of SF1 at Ser20 inhibits pre-spliceosome formation, presumably due to a failure to interact with U2AF65.

Ser20 is required for SF1 function

The role of Ser20 for SF1 activity was further analysed with SF1 proteins carrying Ser20 mutations. SF1–C4, SF1–C4–S20T and SF1–C4–S20A were incubated with PKG in the presence or absence of ATP and reacted with GST–U2AF65. Mutation of Ser20 to Thr severely reduced the interaction, and binding of SF1 to U2AF65 was virtually abolished with the Ala20 mutant protein (Figure 8A). Again, phosphorylation of SF1–C4 and SF–C4–S20T inhibited the interaction with U2AF65. Thus Ser20 is essential for the binding of SF1 to U2AF65.

The mutant proteins were tested for their activity in pre-spliceosome assembly. When 2 or 20 ng of SF1–C4–S20A were added to the reaction, only background levels of complex A were observed (Figure 8B, lanes 7 and 8). Addition of 100 ng of SF1–C4–S20A resulted in the assembly of complex A, but the efficiency was reduced (to 57%) when compared with SF1–C4 (Figure 8B, lanes 6 and 9). Mutant protein SF1–C4–S20T was active at all concentrations tested (Figure 8B, lanes 10–12); however, the efficiency of complex formation was lower than that observed with the SF1–C4 control (ranging from 15 to 88%). We conclude that the activity in pre-spliceosome assembly of the mutant proteins reflects the efficiency of their interactions with U2AF65. It might be surprising that at high concentrations (100 ng) both mutant proteins allow for the assembly of complex A, while U2AF65 binding has been severely compromised. However, interactions of SF1 with proteins other than U2AF65 most likely contribute to the association of SF1 with the spliceosome (Abovich and Rosbash, 1997; Bedford *et al.*, 1998). Our results demonstrate that both PKG phosphorylation of SF1 at Ser20 and mutation of this residue interfere with the interaction between SF1 and U2AF65 and result in reduced levels of pre-spliceosome assembly. This explains the requirement of the N-terminal 28 amino

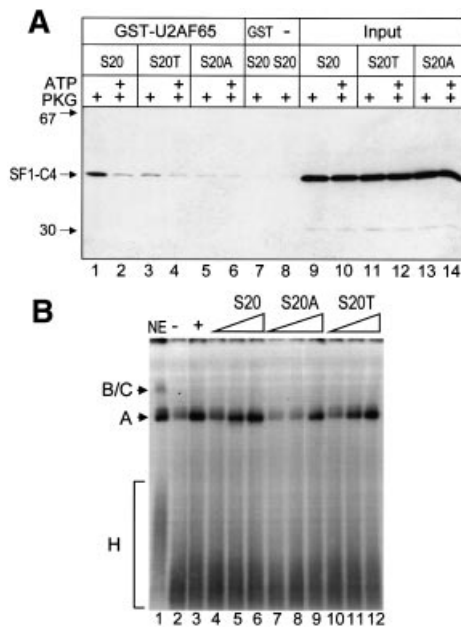


Fig. 8. Mutation of Ser20 in SF1 inhibits its interaction with U2AF65 and pre-spliceosome assembly. **(A)** Effect of Ser20 mutations on the SF1-U2AF65 interaction. SF1-C4 proteins carrying the wild-type Ser20 (lanes 1, 2, 7, 8, 9 and 10) and mutant Thr-20 (lanes 3, 4, 11 and 12) or Ala20 (lanes 5, 6, 13 and 14) were incubated with PKG, in the presence or absence of 1 mM ATP. Proteins were added to glutathione-agarose beads coated with GST-U2AF65 (lanes 1–6) or GST (lane 7), or to glutathione-agarose alone (lane 8). After washing, bound proteins were eluted and detected as in Figure 7. Proteins in lanes 9–14 are the total amount of proteins used in lanes 1–8 (input). **(B)** Effect of Ser20 mutations on pre-spliceosome assembly. Splicing complexes were assembled in the presence of HeLa cell nuclear extract (lane 1) or partially purified splicing components (lanes 2–12). The reactions contained the following additions: buffer (lane 2), SF1 isolated from HeLa cells (lane 3), 2, 20 and 100 ng of SF1-C4 (lanes 4–6), SF1-C4-S20A (lanes 7–9) or SF1-C4-S20T (lanes 10–12). Splicing complexes were analysed as in Figure 7.

acids of SF1 for contacts with U2AF65 and splicing complex formation (Rain *et al.*, 1998).

Discussion

Splicing takes place in the active spliceosome, a dynamic complex of proteins and snRNPs that assembles in a stepwise manner on the pre-mRNA. The initial stage of spliceosome assembly involves an interaction between the two splicing factors SF1 and U2AF65. Here we show that SF1 is phosphorylated by PKG on Ser20, which blocks its interaction with U2AF65 and inhibits assembly of spliceosomal complex A. The ability of SF1 to interact with U2AF65, but not with RNA, was found to be regulated by PKG phosphorylation, but not by PKA. The phosphorylation of SF1 by PKG is the first indication that pre-mRNA splicing may be regulated by cGMP-PKG signalling.

Several criteria were used to identify rat brain P80 as SF1. Amino acid sequencing of four Lys-C-derived peptides revealed 100% identity with SF1 and antibodies raised against synthetic peptides of SF1 specifically recognized P80. Comparison of SF1 and P80 phosphorylation *in vitro* revealed that both were phosphorylated in a similar site by phosphopeptide mapping. The PKG phosphorylation site in SF1 was determined to be Ser20 by direct

sequencing of proteolytic products combined with the development of an improved method of chemical conversion of phosphoserine to *S*-propylcysteine. The site was confirmed by kinetic analysis of phosphorylation of the synthetic peptide SF1_{15–25}, which contained Ser20 as the only Ser residue. The sequence context of the phosphate acceptor site in SF1 is KKRKRSRWN, which agrees well with the motif necessary for efficient PKG phosphorylation determined in studies with peptide libraries on cellulose papers (Tegge *et al.*, 1995). Those studies revealed the minimal motif RKxS/T and highlighted a key role for additional basic amino acids on either side of the RK for optimal V_{max} . The PKG phosphorylation site was determined with recombinant SF1 that lacked the C-terminal proline-rich region and sequences specific to different SF1 isoforms (Krämer *et al.*, 1998 and references therein), raising the possibility that Ser20 might not be the only phosphate acceptor in SF1. Two observations argue against this. First, when the phosphorylation of native and recombinant SF1 was compared by phosphopeptide mapping with V8 protease, a major 16 kDa product was observed with both proteins, suggesting phosphorylation in the same region. PKG failed to phosphorylate SF1-N1a (amino acids 29–370) or a Ser20 to Ala mutation in SF1-C4 (amino acids 2–320), demonstrating the presence of a single PKG phosphorylation site in the N-terminal half of the protein. Secondly, no consensus PKG-I phosphorylation motifs are found in the regions of SF1 that are absent from the recombinant proteins used in this study. Therefore Ser20 is the only phosphorylation site for PKG-I in SF1.

SF1 phosphorylation revealed a high degree of selectivity for PKG. Only four other relatively specific PKG substrates have been previously identified and had their phosphorylation sites sequenced (Wang and Robinson, 1997). SF1 did not serve as an *in vitro* substrate for PKC, cdc2, c-src or CaMPK-II, although potential phosphorylation sites for some of these enzymes are found in SF1. Moreover, SF1 was a poor substrate for PKA, the protein kinase that is most closely related to PKG (Wang and Robinson, 1997). This kinase selectivity was emphasized when the kinetics of phosphorylation of SF1_{15–25} were compared with those of PL_{8–21}. Whereas PKG and PKA phosphorylated PL_{8–21} with very similar kinetics (Kemp and Pearson, 1991; Table I), SF1_{15–25} was a much better substrate for PKG than for PKA. PKG and PKA are known to show overlapping substrate specificities and the canonical PKA phosphorylation site (RRxS/T) is also recognized by PKG (Tegge *et al.*, 1995). Therefore, the minimal motif determined for optimal PKG phosphorylation, RKxS/T, does not account for kinase selectivity and little information is available regarding sequences that may contribute to this. The presence of a Phe C-terminal to the phosphorylation site has been suggested as a negative determinant for PKA phosphorylation (Colbran *et al.*, 1992). SF1 also contains an aromatic amino acid (Trp) in a similar position, but whether this residue accounts for kinase selectivity remains to be tested. In contrast, other substrates for PKG that exhibit this selectivity, VASP and G-substrate, do not have aromatic amino acids C-terminal to the phosphate acceptor site (Wang and Robinson, 1997 and references therein).

SF1 is phosphorylated *in vivo* after appropriate stimula-

tion of the cGMP-PKG signalling pathway. Immunoprecipitates from lysates of ^{32}P -labelled intact neuronal cells contained very low levels of phosphorylated SF1, suggesting that the protein normally predominates in its dephospho-form in these cells. Stimulation of cells with a cGMP analogue increased phosphorylation, while a cAMP analogue had no effect. Phosphorylation site-specific antibodies also revealed that elevated phosphorylation occurred on Ser20, although *in vivo* phosphorylation at additional sites cannot be ruled out at this stage. Therefore, SF1 is most likely a target for PKG signalling *in vivo*. SF1 exists in several isoforms which share the N-terminal 448 amino acids, but vary in the length of the proline-rich region and their C-termini (Krämer *et al.*, 1998 and references therein). Therefore all known isoforms contain Ser20 and we predict that they may all be targets for PKG-I. Notably, only the 80 kDa form of SF1 and a 76 kDa non-specific protein were immunoprecipitated from ^{32}P -labelled cells, but since the latter band was not immunoreactive for SF1 it was not considered further as a potential splice variant. The 80 kDa band observed in the immunoprecipitates may contain more than one SF1 isoform or smaller forms of SF1 may not be expressed in the B35 cell line.

PKG phosphorylation of SF1 blocked its ability to bind to U2AF65 and inhibited pre-spliceosome assembly. Another known property of SF1, RNA binding, was unaffected. SF1 and U2AF65 bind independently of each other to the branch site and the polypyrimidine tract, respectively (Zamore *et al.*, 1992; Abovich and Rosbash, 1997; Berglund *et al.*, 1997). Upon interaction the proteins cooperate to form a ternary complex with the pre-mRNA, and the affinity of SF1 and U2AF65 for the RNA is increased several fold (Berglund *et al.*, 1998a). The results that the assembly of complex A is severely decreased in the presence of phosphorylated SF1 or SF1-C4-S20A demonstrate that the contacts between SF1 and U2AF65 are crucial for the incorporation of the U2 snRNP into the spliceosome and thus for the initial recognition of the branch site by SF1.

Phosphorylation of Ser20 introduces two negative charges into a highly positively charged region of SF1 which may explain the block in protein-protein interaction. However, mutation of Ser to Thr or Ala also compromised the interaction between SF1 and U2AF65, suggesting that the nature of Ser20 itself is an essential feature of the binding site. Ser20 and neighbouring sequences are well conserved between human, *Drosophila* and *Caenorhabditis elegans* SF1 (R.Mazroui and A.Krämer, manuscript in preparation), but not in *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*. Thus, interactions between SF1 and U2AF65 may rely on the same principle in flies and worms. In addition, the conservation of these sequences suggests the possibility of PKG regulation of SF1 activity in these species. Although Ser20 is located at or near the U2AF65 interaction site in SF1, it may not be the only region responsible for binding. A two-hybrid screen between yeast homologues of SF1 and U2AF65 defined an additional interaction region of ~100 amino acids immediately N-terminal to the KH domain, which are highly conserved during evolution (Rain *et al.*, 1998; R.Mazroui and A.Krämer, manuscript in preparation). It is possible that this region

also contributes to the interaction between human SF1 and U2AF65. Whether sequences corresponding to Ser20 in human SF1 that are not well conserved in the yeast SF1 homologues are required for interactions with U2AF65 homologues remains to be tested.

How could cGMP-PKG regulate splicing? Protein kinases that phosphorylate the SR or SR-like proteins, such as SRPK1, SRPK2 and Clk/Sty, are normally found in the nucleus and are implicated in regulating protein-protein and protein-RNA interactions during the course of the splicing reaction (Xiao and Manley, 1998 and references therein). In contrast, PKG-I is a cytoplasmic protein, which makes it unlikely that it constitutively modulates SF1 activity during the spliceosome cycle. Upon stimulation of cells with cGMP, a cryptic nuclear localization signal (NLS) in the ATP binding domain of the protein is exposed, and PKG translocates to the nucleus (Gudi *et al.*, 1997). Immunofluorescence studies confirmed that PKG is present in B35 neuronal cells and is detected in the nucleus when the cells are stimulated with a cGMP, but not cAMP analogue (X.Wang and P.J.Robinson, unpublished results). In agreement with this observation, SF1 phosphorylation was highly increased on Ser20 after stimulation with cGMP. These results have three implications. First, phosphorylation is likely to be mediated by nuclear PKG-I, because SF1 shows an intranuclear distribution typical of many splicing components (D.Nesic and A.Krämer, unpublished results). Phosphorylation by cytoplasmic PKG would be expected if SF1 shuttled between the nucleus and the cytoplasm, as observed for various hnRNP proteins and a subset of SR proteins (Nigg, 1997; Cáceres *et al.*, 1998). In this respect we note that Ser20 is immediately adjacent to a putative NLS in SF1, which raises the possibility that PKG phosphorylation also regulates the intracellular localization of SF1. Secondly, in unstimulated cells we do not expect a regulation of SF1 activity by PKG. However, upon stimulation of cGMP signalling, splicing would be negatively regulated by PKG through interference with the SF1-U2AF65 interaction and spliceosome assembly. Thirdly, we do not expect that PKG will be present in all cells, which limits this regulatory pathway to those cells which express PKG. PKG is found in a wide variety of cell types in low levels, and in high levels in a limited number of cells like Purkinje neurons or smooth muscle cells. However, this does not preclude another unidentified cellular protein kinase from regulating SF1 in cells which do not express PKG.

PKG or cGMP signalling pathways have been linked to gene expression at the transcriptional level (Gudi *et al.*, 1997), but effects on pre-mRNA splicing have not been previously reported. It is known that alternative splice-site selection can be modulated by signal transduction pathways. For example, levels of mRNA for PKC β I in BC3H-1 myocytes quickly decline with insulin treatment, and concomitantly mRNA and protein levels increase for its alternatively spliced variant PKC β II (Chalfant *et al.*, 1995). High levels of phorbol ester mimic insulin action in switching alternative splicing between these transcripts. In another study, it has been demonstrated that CD44 alternative splicing is regulated *in vivo* by signalling through synergistic action of PKC and calcium (König *et al.*, 1998). These studies demonstrate that cell signalling pathways can influence alternative splicing, but the protein

kinases involved or their final targets are not known. Our results highlight a new role for PKG in splicing by regulating the association of SF1 with U2AF65 in a phosphorylation-dependent manner and thereby controlling early steps in spliceosome assembly. The increase in SF1 phosphorylation by cGMP analogues in intact cells provides a first hint that splicing may also be modulated by PKG *in vivo* when cells are appropriately activated.

Materials and methods

Materials

[γ - 32 P]ATP (3000 Ci/mmol) was from Amersham. L-phosphatidylserine (PS), 1,2-diolein, cAMP, cGMP, anti-polyHis antibodies and *Staphylococcus aureus* V8 protease were from Sigma. Leupeptin, calmodulin, histone H1, protein kinase CK2 (human, recombinant), p34^{cdc2}/cyclin B (human, recombinant) and c-src were from Calbiochem. 8-pCPT-cGMP and 8-pCPT-cAMP were from Biolog Life Science Institute (Bremen, Germany). Okadaic acid was from Gibco-BRL. Rat brain CaMPK-II was a generous gift from Dr John Rostas, University of Newcastle, NSW, Australia. Synthetic peptide substrates were SF1₁₅₋₂₅ (KKRKRSRWNQD) from Auspep, Melbourne; PL₈₋₂₁ (TRSAIRRASTIEMP) from Macromolecular Resources, CO; and the Walsh inhibitor of PKA (PKI₅₋₂₄ TTYADFIASGRTGRRNAIHD) from the Peptide Synthesis Group of Louisiana State University. All peptides were purified by HPLC to >90% purity.

Protein purification

P80 was purified from a fraction enriched in Ca²⁺-sensitive lipid-binding proteins (Wang and Robinson, 1995) prepared from 40 rat brains. Proteins in this fraction were precipitated by (NH₄)₂SO₄ to 80% saturation, dialysed against buffer A [20 mM Tris-HCl, 1 mM dithiothreitol (DTT), 1 mM EDTA, 0.1% Tween 80 pH 7.7] and applied to a Q-Sepharose high-performance anion exchange column (10 × 1.6 cm, Amersham Pharmacia Biotech). After washing in buffer A, bound proteins were eluted with a 0–400 mM NaCl gradient at 2 ml/min and sixty 2 ml fractions were collected. Aliquots of every second fraction were phosphorylated (see below) and fractions containing P80 were pooled. The pool was diluted to adjust the NaCl concentration to <40 mM and the pH to 7.0, and applied to an S-Sepharose high-performance cation exchange column (10 × 1.6 cm, Amersham Pharmacia Biotech) which was pre-equilibrated with buffer A, pH 7.0. After extensive washing, bound proteins were eluted with 0–400 mM NaCl gradient at 1 ml/min and sixty 1 ml fractions were collected. Aliquots of the column fractions were phosphorylated and fractions containing P80 were pooled, concentrated and desalted with an ultra free-4 centrifugal filter device (Millipore, MA) and stored at –20°C in buffer A, pH 7.0.

The catalytic subunit of PKA and protein kinases C (a mix of PKC α , β and γ) were purified from bovine lung and rat brain as previously described (Robinson *et al.*, 1993). Bovine lung PKG-I was purified to homogeneity using DEAE cellulose and cAMP-affinity columns with additional steps as described (Robinson *et al.*, 1993) and was stored at –70°C in 10% glycerol with 0.05% Tween 80.

Protein phosphorylation

Phosphorylation of brain extracts or column fractions was performed in a final volume of 80 μ l. The reaction contained 30 mM Tris-HCl pH 7.4, 1 mM EGTA, 1 mM MgSO₄, 40 μ M ATP, 0.125 μ Ci/ μ l [γ - 32 P]ATP and 0.05% Tween 80. Where appropriate, the following activators of protein kinases were included: PKC—1.2 mM CaCl₂ (generating 200 μ M free Ca²⁺), 40 μ g/ml PS and 4 μ g/ml diolein; CaMPK-II—10 μ g/ml of calmodulin and 1.2 mM CaCl₂; or PKG—10 μ M cGMP. A peptide derived from the Walsh inhibitor of PKA, PKI (0.01 mg/ml), was routinely included in the assays for PKG and PKC to prevent cross-activation by cGMP of any PKA potentially present in column fractions. After prewarming the tubes to 30°C for 5 min, reactions were initiated by addition of 10 μ l of ice-cold brain samples, followed immediately by the purified protein kinase (20–40 ng/reaction). Reactions were terminated after 5 min by addition of 50 μ l of SDS sample buffer and rapid freezing of samples on dry ice, as previously described (Robinson and Dunkley, 1983). Phosphoproteins were detected by gel electrophoresis and autoradiography (Robinson and Dunkley, 1983) using 7.5–15% acrylamide linear gradients and 20 cm gels (Protean II system, Bio-Rad).

Phosphopeptide maps using V8 protease were performed on native SF1 and recombinant SF1 excised from dried gels as described (Dunkley *et al.*, 1986). Phosphoamino acid analysis of 32 P-labelled proteins excised from polyacrylamide gels was performed under the conditions described previously (Robinson, 1991).

Protein kinase activity was determined in the presence of 30 mM Tris-HCl pH 7.4, 1 mM EGTA, 200 μ M ATP, 2 μ Ci [γ - 32 P]ATP, 10 mM MgSO₄ in 40 μ l final reaction volumes. Incubations were for 5 min at 30°C using the synthetic peptide substrates PL₈₋₂₁ (Kemp and Pearson, 1991) or SF1₁₅₋₂₅ at 0.1 mg/ml. The peptides were used over a broad concentration range (0.003–0.3 mg/ml) for determination of substrate kinetics. Reactions were initiated by addition of 40 ng of PKG or 20 ng of the catalytic subunit of PKA. These amounts of PKG and PKA were determined in prior experiments to phosphorylate PL₈₋₂₁ to the same level, since this substrate has the same V_{max} for both protein kinases (Kemp and Pearson, 1991). Reactions were terminated by the addition of 75 mM phosphoric acid and aliquots were spotted onto Whatman P81 paper, washed 3 times for 10 min each in 75 mM phosphoric acid, dried and counted by liquid scintillation techniques (Robinson, 1992). Kinetic constants K_m and V_{max} were determined with the PC program Enzfitt (written by Robin Leatherbarrow, Sigma).

Amino acid sequencing and phosphorylation site determination

For amino acid sequencing P80 was excised from a dried Coomassie Blue-stained polyacrylamide gel, digested with Endo-Lys-C (from *Achromobacter lyticus*) and the resultant peptides were separated by HPLC. Four major peaks were sequenced with an Applied Biosystems Procise sequencer model 494-HT.

To determine the phosphorylation site, recombinant SF1-C4 (50 μ g) was phosphorylated with PKG and [γ - 32 P]ATP as above (except that the incubation volume was increased to 500 μ l and the incubation time was 30 min) prior to digestion with Endo-Lys-C and separation of resultant peptides by HPLC. The labelled fraction was detected by Cerenkov counting. Two peaks of radioactivity were detected, a major peak (peptide 1, SF1₁₉₋₂₈ RSRWNQDTME) and a minor peak. Since we had determined that the only phosphorylated amino acid in SF1 was phosphoserine (see Results), to confirm which amino acid in each fraction was phosphorylated, a new method to detect phosphoserine was developed. Phosphoserine was converted to its thioether derivative, S-propylcysteine, using a method which combines the addition of an alkanethiol to the α - β unsaturated dehydroalanine (Reynolds *et al.*, 1994) generated by the Ba²⁺-catalysed β -elimination of phosphoserine (Byford, 1991). The column fractions containing the 32 P label were lyophilized and incubated with 50 μ l of freshly prepared ethanolic solution saturated with barium hydroxide containing 20% 1-propanethiol (350 μ l ethanol, 110 μ l 1-propanethiol, 65 μ l 5 M NaOH, 60 μ l 0.1 M BaCl₂; excess Ba(OH)₂ precipitates were removed by centrifugation) for 1 h under argon at 50°C. The reaction was terminated by the addition of 10 μ l of 10% trifluoroacetic acid (TFA) and the mixture was dried in a centrifugal evaporator. The residue was redissolved into 10% aqueous TFA/50% acetonitrile (50 μ l) and re-evaporated to remove residual thiol. The peptides were dissolved into the same TFA-acetonitrile mixture (15 μ l) and dried onto 8-mm TFA-activated glass fibre micro-filters (Perkin Elmer) which had been pre-treated with 1.5 mg of BioBrene Plus (Perkin Elmer). The samples were subjected to N-terminal sequence analysis as above and PTH-S-propylcysteine was observed to elute, fully resolved, after PTH-leucine.

DNA constructs

SF1 deletion mutants containing N-terminal His₆-tags have been described previously (Rain *et al.*, 1998) and are shown schematically in Figure 2A. Point mutations at Ser20 were introduced by PCR (Rain *et al.*, 1998) with complementary primers corresponding to the cDNA sequence of amino acids 15–25 of SF1. The Ser codon AGC was changed to ACC to generate SF1-C4-S20T and to GCC for SF1-C4-S20A. Amplified DNA was transformed into XL1-Blue cells and mutations were confirmed by sequencing (T7 DNA sequencing kit, Pharmacia). Mutant SF1 plasmids were transformed into the *Escherichia coli* strain TOP10 (Invitrogen). Recombinant SF1 proteins were expressed and purified on Talon affinity resin as described (Rain *et al.*, 1998). Purified proteins were dialysed against buffer D (Dignam *et al.*, 1983). A plasmid encoding GST-U2AF65 (Zamore *et al.*, 1992) was kindly provided by M.Green and J.Valcárcel. Expression of GST and GST-U2AF65 was performed as described (Rain *et al.*, 1998). A plasmid encoding ASF/SF2 was a gift from J.Manley and the protein was expressed and purified as described (Ge *et al.*, 1991).

GST binding assays

Protein phosphorylation was performed as described above, but in the presence of 1 mM ATP and omitting the [γ - 32 P]ATP. Mock-phosphorylation was performed in the absence of ATP. SF1 proteins were separated from PKG by purification on Talon affinity resin prior to interaction with U2AF65 (Rain *et al.*, 1998). Proteins were separated in 12% SDS-polyacrylamide minigels and blotted onto Protran membranes (Schleicher and Schüll; Kyhse Andersen, 1984). SF1 was detected by incubation with anti-poly-His antibodies (1:3000 dilution) and anti-rabbit secondary antibodies (1:2000 dilution, DAKO) followed by incubation with enhanced chemiluminescence (ECL) reagents (Amersham).

Spliceosome assembly

Splicing complexes were assembled on a synthetic 32 P-labelled pre-mRNA substrate derived from the adenovirus major late transcription unit, as described previously (Krämer and Utans, 1991). Reactions contained partially purified SF3a, SF3b, U2AF, U1 and U2 snRNPs and SF1 isolated from HeLa cells (Krämer, 1992) or recombinant SF1-C4 and its mutated variants. The SR proteins that are essential for the reaction are present in the snRNP fractions. Okadaic acid (1 μ M) was added to inhibit endogenous protein phosphatases. Phosphorylation of SF1-C4 and Talon purification were done as above except that PKG was omitted from the mock phosphorylation reaction. A reaction performed in HeLa cell nuclear extract (Dignam *et al.*, 1983) served as a control for spliceosome assembly. Splicing complexes were separated in a non-denaturing 4% polyacrylamide gel.

Antibodies

SF1 antibodies were raised in sheep against two different synthetic peptides: SF1₁₋₁₈ MATGANATPLDFPSKRRK and SF1₈₇₋₁₀₅ YNSEGKRLNTRFRTRKRL. The peptides were conjugated to diphtheria toxoid as a carrier and were used to immunize the same sheep. Phosphorylation site-specific polyclonal antibodies to phospho-Ser20 were also raised in sheep against a synthetic peptide encompassing the phosphorylation site, and including a phosphorylated serine residue at the time of peptide synthesis [SF1₁₇₋₂₄ RKRS(P)RWNQ, with a cysteine added at the C-terminus for conjugation to diphtheria toxoid]. Peptides were synthesized by Chiron (Melbourne, Australia). Immunoblotting was performed by chemiluminescent detection (Pierce SuperSignal).

Tissue culture, intact cell labelling and immunoprecipitation

B35 cells were grown in six-well plates in DME with 10% fetal calf serum and were differentiated with 100 μ M dibutyryl-cAMP for 2 days. The cells were washed twice with phosphate-free medium and labelled with 500 μ Ci/ml 32 P_i for 1.5 h. After washing with phosphate-free medium to remove free 32 P_i the cells were divided into three aliquots: (i) control, without additions; (ii) stimulated by the cell membrane permeable PKG agonist 8-*p*-chloro-phenylthio-cGMP (8-pCPT-cGMP, 200 μ M); and (iii) stimulated by the PKA agonist 8-pCPT-cAMP (200 μ M). The cells were stimulated for 30 min, washed and lysed with 20 mM Tris, 1 mM DTT, 1% Triton X-100, 7 μ g/ml leupeptin, 1 mM EGTA and 1 mM EDTA pH 7.4. SF1 was immunoprecipitated from the 32 P-labelled cell lysate as described (Robinson *et al.*, 1993) and detected in gels by autoradiography.

For the detection of the *in vivo* phosphorylation site, cells were treated the same way but were not radiolabelled. Prior to immunoprecipitation the cell lysate was applied to a Q-Sepharose column (1 ml of resin), washed with buffer A and proteins were batch-eluted with buffer A plus 300 mM NaCl. SF1 was immunoprecipitated from the eluates and proteins were run on SDS-PAGE minigels and transferred to nitrocellulose. In these experiments the SF1 antibodies were first purified on protein G-Sepharose before immunoprecipitation. The blots were probed with the same anti-SF1 antibodies, stripped with 0.2 M NaOH for 30 min at room temperature and re-probed with anti-phospho-Ser20 antibodies. Protein G-Sepharose-purified antibodies were used for both immunoprecipitation and blotting. The Q-Sepharose step eliminated most of the background bands detected with the antibodies.

Acknowledgements

The authors wish to thank Eric Reynolds for the suggestion for the phosphoserine conversion to *S*-propylcysteine, John Rostas for the purified CaMPK-II, John Haycock for advice on the phosphorylation site antibodies, Michael Green and Juan Valcárcel for the gift of GST-U2AF65, Jim Manley for the plasmid encoding ASF/SF2 and Peter Rowe for comments on the manuscript. This work was supported by

grants from the Australian National Health and Medical Research Council (NHMRC to P.J.R. and X.W.), the Swiss National Science Foundation and the State of Geneva (A.K.) and the Australian Research Council (P.J.M.).

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Received March 2, 1999; revised and accepted July 2, 1999