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MAJOR PHOSPHORYLATION OF SF1 ON ADJACENT SER-PRO MOTIFS ENHANCES INTERACTION WITH U2AF⁶⁵

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Summary

Protein phosphorylation ensures the accurate and controlled expression of the genome, in particular by regulating the activities of pre-mRNA splicing factors. Here we report that Splicing Factor 1 (SF1), which is involved in an early step of intronic sequence recognition, is highly phosphorylated in mammalian cells on two serines within an SPSP motif at the junction between its U2AF⁶⁵ and RNA binding domains. Interaction of the protein kinase KIS with SF1 through its “U2AF Homology Motif” (UHM) domain is necessary for its efficient phosphorylation of SF1 on these sites. Importantly, phosphorylation of SF1 on the SPSP motif leads to increased binding to U2AF⁶⁵ and enhanced formation of the ternary U2AF⁶⁵-SF1-RNA complex, suggesting an important role of this phosphorylation event in the function of SF1 and possibly in the structural rearrangements associated with spliceosome assembly and function.

Keywords

Protein phosphorylation; RNA splicing; U2AF-homology-motif

Introduction

The expression of the genome requires the precise and controlled removal of intervening sequences within pre-messenger RNAs (pre-mRNA splicing). Assembly of the active spliceosome requires successive rearrangements, including the entry and exit of molecular partners (reviewed in Ref. [1]). Phosphorylation events are likely molecular switches to control these conformational changes. Indeed, experiments with phosphatase inhibitors, purified phosphatases and nonhydrolysable ATP analogues have shown that multiple phosphorylation and dephosphorylation events are required for spliceosome assembly and splicing [2-4]. Among the best-characterized of the phosphorylated splicing factors are the SR protein family (reviewed in Ref.[5]), whose intranuclear distribution and activity is influenced by phosphorylation. Several specific kinases have been identified for SR protein phosphorylation, including SRPK1, SRPK2 [6,7], and Clk/Sty [8]. SF3b155/SAP155, an integral spliceosome

component and substrate of cyclin E/CDK2 [9], is a non-SR protein whose phosphorylation state is regulated during the splicing process [10].

Splicing factor 1 (SF1) was independently identified as necessary for spliceosome assembly by *in vitro* reconstitution assays with protein fractions from HeLa nuclear extracts [11], and in a synthetic lethality screen with the yeast homologue of the splicing factor U2AF⁶⁵ (Mud2p) [12]. Initially, SF1 binds to the branch point pre-mRNA consensus sequence (BPS) near the 3' splice site [13], and facilitates binding of the essential splicing factor U2 Auxiliary Factor large subunit (U2AF⁶⁵) to the adjacent poly-pyrimidine tract pre-mRNA consensus (Py-tract) [14] by direct protein-protein interactions [15]. Next, SF1 is displaced from the spliceosome by the ATP-dependent entry of the U2 small nuclear ribonucleoprotein particle (snRNP), whose SF3b155/SAP155 protein subunit interacts with U2AF⁶⁵ [16] and RNA component (U2 snRNA) anneals with the BPS [17,18]. This first ATP-dependent step of 3' splice site recognition represents a critical juncture for regulation of pre-mRNA splicing. The protein kinase PKG is a potential regulator of this step, by inhibiting the SF1/U2AF⁶⁵ complex upon phosphorylation of a conserved SF1 serine (Ser20) within its U2AF⁶⁵ interaction domain [19].

The solution structure of the minimal SF1/U2AF⁶⁵ complex [20] reveals that the U2AF⁶⁵ domain belongs to a subclass of RNA recognition domains with specialized features for protein-protein interactions named U2AF Homology Motifs (UHM), based upon structural homology with the U2AF small subunit (U2AF³⁵) [21,22]. Based on critical UHM features for interaction with the peptide ligands, diverse UHM-containing proteins were identified, including the mammalian protein kinase KIS [20,21]. The KIS polypeptide is organized into a kinase core followed by a C-terminal UHM motif [23-25], and preferentially phosphorylates Ser-Pro sites *in vitro* [26]. A role for KIS during control of cell-cycle division is supported by the phosphorylation of the nuclear CDK inhibitor p27^{kip1} [27] and the observation that KIS mRNA levels are misregulated in neurological tumors [23].

We report here that SF1 is phosphorylated on two major adjacent SerPro motifs (hereafter called the SPSP motif). We show that the protein kinase KIS can interact with SF1 through its UHM domain and efficiently phosphorylate SF1 on both serines of this SPSP motif, and therefore likely participates in controlling the phosphorylation state of SF1. Finally, SF1 SPSP motif phosphorylation enhanced its interaction with U2AF⁶⁵ and formation of a ternary complex with U2AF⁶⁵ and a model 3' intronic sequence, suggesting the importance of these major phosphorylations in regulating SF1 function.

Results

Part I : Phosphorylation of SF1 on serines 80 and 82 *in vitro* and *in vivo*

The presence of two adjacent SerPro motifs (aa 80-83, hereafter called SPSP motif) in a highly conserved region of splicing factor SF1 suggested that these serine residues could be targets of the proline directed kinase KIS whose UHM domain is a putative ligand for SF1. Furthermore, using pull-down experiments, we observed that KIS binds efficiently to a fragment of SF1 containing the SPSP motif (human SF1 residues 1-255, hereafter called SF1f) *in vitro* (Fig. 1). Interestingly, KIS binds SF1f as efficiently as U2AF⁶⁵ lacking its RS domain (U2AF⁶⁵ΔRS) (Fig. 1, compare lanes 7 and 12). In our conditions, binding of U2AF⁶⁵ΔRS to SF1f was about twofold less than that of U2AF⁶⁵ (lane 11) suggesting an unsuspected contribution of the RS domain of U2AF⁶⁵ to SF1f binding. The possibility that the RS domain is necessary to stabilize the structure of the C-terminal domain could also explain this difference. A complete or a partial deletion of the UHM domain of KIS or mutations of two acidic residues (E341 and D342) to lysine in the putative A helix of the KIS UHM whose counterparts in U2AF⁶⁵ have been shown to be required for binding to SF1 severely reduced

binding to SF1f (lanes 8, 9 and 10). Therefore, when compared to U2AF⁶⁵, KIS binds efficiently to SF1f and this interaction requires structural features of its UHM that are shared with U2AF⁶⁵.

Kinase assays with recombinant KIS showed an efficient phosphorylation of SF1f, as evidenced by ³²P phosphate incorporation and a marked shift of the SF1f band on SDS PAGE (Fig. 2A). The interaction of the UHM domain of KIS with SF1 appears important for efficient phosphorylation as deletions within the UHM domain of KIS prevented SF1f phosphorylation (Fig. 2B, lanes 3 and 4). In all conditions tested, phosphate incorporation never exceeded an evaluated stoichiometry of two phosphates for one SF1f molecule indicating that phosphorylation occurs on two residues. By mass spectrometry analysis, following tryptic digestion of SF1f phosphorylated to high stoichiometry (over 1.5 phosphates per SF1 molecule), we identified masses corresponding to peptide 67-92 and peptide 80-92, each with two phosphates. These peptides contain serines 80 and 82 in the putative KIS target SPSP motif. Phosphorylation velocity decreased by approximately two-fold when using SF1f with serine 80 or serine 82 mutated to alanine (Fig. 2C), indicating that KIS can phosphorylate both sites with similar efficiency whereas the double alanine mutation completely inhibited phosphorylation indicating that no phosphorylation occurs outside the SPSP motif. Altogether, KIS can phosphorylate SF1f on serine 80 and 82 with a high efficiency that particularly relies on the anchoring of its UHM domain to SF1. The respective involvement of KIS and other kinases and phosphatases in controlling the SF1 SPSP motif phosphorylation will need important studies. However interestingly, in the following report, KIS reveals itself a very powerful tool to demonstrate the major *in vivo* phosphorylation of the SPSP motif of SF1 as well as associated modifications of its interacting properties.

To determine whether the SPSP motif was phosphorylated *in vivo*, we performed metabolic labelling of HEK293 cells overexpressing SF1HL1myc (a major spliced form of SF1 [28], with a myc tag, hereafter SF1myc), followed by immunoprecipitation with antimyc antibody. As shown in Fig. 3A, mutation of SF1 on both serines 80 and 82 (hereafter called 8082A) dramatically reduced phosphate incorporation (about four-fold) showing that the SPSP motif contains major phosphorylation sites of SF1 *in vivo*. Remaining incorporated phosphate corresponds to phosphorylation on other sites like serine 20 which was shown to be phosphorylated *in vivo* [19].

To confirm the major phosphorylation of the SPSP motif, we compared the tryptic phosphopeptide map of SF1f phosphorylated *in vitro* by KIS to that of SF1myc phosphorylated in cells (Fig. 3B, maps 1 and 2). In fact, these peptide maps were highly similar. The tryptic peptide maps presented multiple spots corresponding to incomplete digestion by trypsin as previously observed by mass spectrometry analysis, and to the presence of single and double phosphorylation of the peptides as suggested by our analysis of SF1f phosphorylated *in vitro* by KIS to different extents (data not shown). Co-expression of KIS did not obviously increase the incorporation of radioactive phosphate (Fig. 3A, lane 3), but phosphopeptide mapping revealed its effect to increase the proportion of more acidic peptides (c, d, e) (Fig. 3B, compare maps 2, 3) suggesting that it increases the phosphorylation state of SF1 on the SPSP motif. Coexpression with KIS(54R), a mutation within the kinase active site that suppresses activity [24], had an intermediate effect (Fig. 3B, map 4). This agreed with our unpublished observations that KIS(54R) retains low levels of kinase activity towards SF1, due to the unusually high activity of KIS for this substrate.

The observation that wild type SF1myc migrates slower than the 8082A mutant (compare lanes 1-3 with 4-6 in Fig. 3A) further indicated that the SPSP motif of SF1 is mostly in a phosphorylated state in proliferating HEK293 cells. We tested this possibility by treating nuclear extracts of SF1myc overexpressing cells with alkaline phosphatase. As shown in Fig.

3C, phosphatase treatment induced a faster migration of SF1myc on SDS-PAGE showing that SF1myc is on average highly phosphorylated in proliferating cells. This was also the case for endogenous forms of SF1 in HEK293 (Fig 2C, lower panel) and HeLa cells (not shown).

To summarize, these analyses show that the SPSP motif contains major *in vivo* phosphorylation sites of SF1. In agreement with this result, two recent independent large scale analysis of the phosphoproteome, one in nuclear extract of HeLa cells, the other in WEHI-231 B lymphoma cells, lead to the identification of the same 67-92 peptide of SF1 phosphorylated on both Serines 80 and 82 [29,30]. Altogether, this high level of phosphorylation of its SPSP motif indicates a particular involvement in SF1 function.

Part II: Modulation of SF1 binding properties upon SPSP motif phosphorylation

The SPSP motif lies at the junction between the U2AF⁶⁵ binding N-terminal region of SF1 and its RNA binding domain, suggesting that phosphorylation on these sites might modulate the formation of the SF1-U2AF-RNA complex.

We first tested the effect of phosphorylation on the interaction of SF1 with U2AF⁶⁵ by pull-down experiments. Using GST-SF1f that was phosphorylated by KIS to high stoichiometry (over 1.5 phosphate per SF1f molecule) we observed a two-fold increase in U2AF⁶⁵ and a three-fold increase of U2AF⁶⁵ΔRS binding (Fig. 4). In contrast no increase in binding could be observed with the 8082A SF1f mutant upon treatment with KIS, showing that phosphorylation on the SPSP motif is responsible for U2AF⁶⁵ binding enhancement.

As SF1 and U2AF⁶⁵ have been shown to bind in a cooperative manner to a model 3' splice site RNA, we hypothesised that the modification of SF1 binding to U2AF⁶⁵ could in turn modulate the formation of the SF1-U2AF⁶⁵-RNA ternary complexes. We analysed the formation of this complex by RNA gel-shift. As shown in Fig. 6, the shifts of RNA induced by U2AF⁶⁵, and SF1f plus U2AF⁶⁵ binding were clearly identified as previously described by Berglund and coll. [14]. Interestingly we observed an increase in ternary complex formation (compare Fig. 5A, lanes 3, 4 and 5). In contrast, no major modification of SF1f alone binding to RNA was observed (Fig. 5B). Further analysis using increasing concentrations of SF1f or pSF1f with constant concentration of U2AF⁶⁵ (1.2μM) confirmed the enhancement of ternary complex formation with phosphorylated SF1 (Fig. 5C). This was observed using three different productions of phosphorylated SF1 versus parallel productions of mockphosphorylated SF1. The ratio of the apparent K_d were calculated by linear regression of the Hill plot for four experiments showing an about three fold decrease of apparent K_d for SF1 binding to RNA upon phosphorylation by KIS. To summarize, these results show that phosphorylation of SF1 on the SPSP motif enhances binding to U2AF⁶⁵ and formation of the ternary SF1-U2AF⁶⁵-RNA complex.

Discussion

Importance of the interaction of SF1 with U2AF⁶⁵

A comprehensive set of data indicate the functional importance of the interaction of SF1 with U2AF⁶⁵. In mammals, the interaction between U2AF⁶⁵ and SF1 was demonstrated in two-hybrid, pulldown and farwestern assays [12,14,15]. These interactions involve the highly conserved N-terminus of SF1 and UHM domain of U2AF⁶⁵. In addition, SF1 and U2AF⁶⁵ were shown to be both present in the early spliceosomal complex E [12,31]. SF1 was shown to bind preferentially to the pre-mRNA branchpoint sequence [13,32] while U2AF⁶⁵ interacts with the close pyrimidine tract and its RS domain contacts the branchpoint [33-36]. Furthermore the interaction of U2AF⁶⁵ and SF1 with the branchpoint region were found to be cooperative [14]. Finally structural basis for this interaction were determined [20].

The SF1-U2AF⁶⁵ interaction appears highly conserved through evolution. In *S. pombe*, SF1 and the U2AF subunits orthologs U2AF⁵⁹ and U2AF²³ form a stable complex [37]. In *S. cerevisiae* the SF1 ortholog Msl5p is highly conserved while the U2AF⁶⁵ ortholog Mud2p shows a sequence conservation restricted to the UHM domain involved in its interaction with SF1. Like their mammalian counterparts, Msl5p and Mud2p interact and are recruited early in spliceosome assembly [38,39]. Msl5p has a marked preference for binding to the consensus *S. cerevisiae* branchpoint sequence [13] and Mud2p requires an intact branchpoint region for binding [38,40]. Furthermore *MSL5* was identified by virtue of its genetic interaction with *MUD2* [12].

In this context, our finding that the SF1-U2AF⁶⁵ interaction can be enhanced by phosphorylation is most probably of importance for SF1 function. Importantly, SF1 is thought to perform an essential conserved function for cell viability. Actually, in HeLa cells, siRNA depletion of SF1 leads to cell death [41] and in *S. cerevisiae* *MSL5* disruption is lethal [12].

However, despite the well-characterized role of U2AF⁶⁵ in splicing, the strong evidence for the SF1-U2AF⁶⁵ interaction taking place early during spliceosome formation and the fact that SF1 is necessary for spliceosome assembly in *in vitro* reconstitution assays with protein fractions from HeLa nuclear extracts [42], experimental perturbations of SF1 did not lead to major impairment of splicing of different pre-mRNA. Actually thorough immunodepletion of SF1 from a HeLa splicing extract only modestly reduced the kinetic of spliceosome assembly [43]. Similarly no differences in splicing *in vitro* for three substrates could be detected when complementing a U2AF⁶⁵ depleted extract with full-length U2AF⁶⁵ or U2AF⁶⁵ lacking its UHM domain, suggesting that the SF1-U2AF⁶⁵ interaction could be dispensable *in vitro* for splicing of at least a subset of pre-mRNA [44]. Using *S. cerevisiae* extracts depleted for SF1 or prepared from temperature-sensitive mutants grown at the non permissive temperature, no splicing defect of a pre-mRNA with consensus splice sites was detected, but splicing of pre-mRNA with weakened splice site sequences was reduced, suggesting that partial depletion of SF1 function at least affects splicing efficiency [39,45]. Finally, SF1 knockdown in HeLa cells using siRNA, although leading to cell death, did not apparently affect splicing of several pre-mRNA nor the levels of a variety of proteins translated from spliced mRNA [41]. The possibility that low levels of remaining SF1 in depleted extracts and in siRNA treated cells are sufficient for its transient catalytic action in prespliceosome assembly on most pre-mRNA has been proposed [39,41,45] thus leaving the possibility that SF1 has an essential role in splicing that could not be evidenced in these different conditions. In the conditions where SF1 function is repressed, a splicing defect of a subset of pre-mRNA for which SF1 is rate limiting, some of which encoding essential peptides has been proposed to explain its effect on cell viability [45]. Alternatively, a cumulative effect of modestly reduced kinetic in splicing of a larger subset of pre-mRNA leading to a deficiency of an essential function could explain the reported results. Finally, functions of SF1 other than splicing have been suggested that could be related to its requirement for cell viability. Namely, SF1 was found to bind transcription factors and to repress transcription in a reporter assay in mammalian cells [46,47]. In *S. cerevisiae*, Msl5p temperature-sensitive mutants at the non permissive temperature presented a pre-mRNA retention defect [45]. Interestingly in this context, U2AF⁶⁵ has been shown to be a shuttling protein and its function in RNA export has been documented [48,49].

In conclusion further work is clearly needed to decipher the function of SF1 in splicing and other processes. Given the strong evidence supporting the conserved SF1-U2AF⁶⁵ interaction, it is likely that the regulations of this interaction by phosphorylation of serine 20 [19] and of the highly conserved SPSP motif might be of major importance for the control of its function (s). Finally the identification of these phosphorylated forms of SF1 opens new possibilities to investigate its function.

Speculations concerning the functional and structural consequences of SPSP motif phosphorylation

The importance of the SF1 SPSP motif phosphorylation was first suggested to us by a very efficient phosphorylation of SF1 by the U2AF⁶⁵ related protein kinase KIS whose C-terminus UHM domain is in fact the closest homolog of the UHM of U2AF⁶⁵. Phosphorylation occurred exclusively on serine residues 80 and 82 within the SPSP motif. Interestingly we found that KIS can interact *in vitro* with SF1 as efficiently as U2AF⁶⁵ΔRS and that its UHM domain is required for binding to and to phosphorylate SF1. KIS appears as a likely candidate for controlling the SF1 SPSP motif phosphorylation and its respective involvement and that of other kinases and of phosphatases in this control *in vivo* is the subject of further investigations.

Next we found that SF1 is on average highly phosphorylated on the SPSP motif in HEK293 cells. This was also the case in HeLa cells and mouse brain (data not shown). In fact the faster migrating unphosphorylated form of SF1 was undetectable without phosphatase treatment of the extracts. The functional significance of this major modification of SF1 was further suggested by the induced modification of its interaction with U2AF⁶⁵. Actually in pulldown experiments, U2AF⁶⁵ recovery on GST-SF1 beads was markedly enhanced when SF1 was phosphorylated by KIS (Fig. 4). In gel-shift experiments with a model 3' splice site RNA, SF1 SPSP motif phosphorylation enhanced the formation of the ternary U2AF⁶⁵-SF1-RNA complex leading to a marked decrease of the apparent K_d of SF1 for RNA in the presence of U2AF⁶⁵ (Fig. 5). Finally, the SPSP motif is highly conserved as well as surrounding residues (Fig. 2D) suggesting that phosphorylation of this motif is a conserved mechanism for SF1 regulation.

In view of the discussed proposed functions for SF1, this phosphorylation event could be important for constitutive or alternative splice site selection by facilitating recognition of weak acceptor sequences, it could participate in the nuclear retention of pre-mRNA or participate in a control of transcription associated with prespliceosome assembly. The observed highly phosphorylated state of SF1 could reflect that SF1 dephosphorylation is transient for example for recycling of SF1 after prespliceosome assembly. It is tempting to speculate that dephosphorylation of SF1, by decreasing its affinity for U2AF⁶⁵, might help the replacement of SF1 by the U2snRNP component SF3b155/SAP155. Interestingly, the fact that phosphorylation occurs at a distance from the U2AF⁶⁵ binding domain suggests that the SPSP motif remains accessible to a regulating phosphatase when SF1 is bound to U2AF⁶⁵ and RNA. This hypothesis is also supported by the fact that phosphorylation within proline rich regions are known to induce structural rearrangements [50] that could therefore propagate up to the U2AF⁶⁵ binding site in the case of SF1. The observation by Rain and coll [15] that two alpha helices are predicted to form on each side of the proline-rich region containing the SPSP motif suggests that structural modifications at the SPSP region by phosphorylation could become amplified by these helical structures, thereby facilitating the SF1-U2AF⁶⁵ interaction. However we cannot exclude that the phosphorylated serine residues directly participate in the interaction with U2AF⁶⁵. Of note, analysis of binding of deleted forms of SF1 showing that residues downstream residue 25 were dispensable for interaction were performed with unphosphorylated SF1 [20]. Our data suggest that the phosphorylated SPSP do not interact with the basic RS domain because binding enhancement upon phosphorylation was also observed with U2AF⁶⁵ΔRS. Further investigations are aimed at dissecting the structural basis for the effect of SPSP motif phosphorylation on the interaction of SF1 with U2AF⁶⁵.

Altogether, the high level of phosphorylation of SF1 on the phylogenetically conserved SPSP motif *in vivo*, together with the associated modifications of its interactions properties strongly suggest a functional implication for SF1 function(s). Further studies will address the questions of whether and how RNA splicing and/or the other potential functions of SF1 in pre-mRNA

retention or transcription repression are regulated by SF1 SPSP motif phosphorylation and by KIS.

Experimental procedures

Plasmids and mutagenesis

Plasmids for expression of human SF1 (residues 1-255) (SF1f) was constructed in the pGEX6P-1 vector (Pharmacia). SF1f contains the U2AF⁶⁵ binding domain and KH-QUA2 domain for BPS recognition. Prior to PCR, the SF1 template was corrected for a Arg19Gly mutation inadvertently present from previous published work [14]. Plasmids pSP64-U2AF⁶⁵ and U2AF⁶⁵ΔRS (lacking aa 25 to 63) [33] for *in vitro* translation were kindly provided by J. Valcarcel. To allow expression of rat KIS from the same vector, an NcoI-XhoI blunt insert from plasmid BSKIS [24] was transferred to pSP64-U2AF⁶⁵ NcoI-EcoRI blunt yielding pSP64-KIS. For expression in mammalian cells, SF1HL1 cDNA was amplified from cDNA of HeLa cells and subcloned in pCDNA3 with a myc tag at the C-terminal (SF1myc in the text). Site-directed mutagenesis was performed using Quickchange protocol from Stratagene.

Protein expression and purification

GST-KIS and mutants were produced as previously described [26]. GST-SF1f was purified by glutathione-affinity chromatography, followed by removal of GST using Precision Protease (Pharmacia) and further purification on SP-sepharose (Pharmacia). Purified fractions were frozen in 200 mM NaCl, 25 mM Hepes pH6.8, 20% v/v glycerol. Plasmid for U2AF⁶⁵ was a gift of M. Green, and HIS-tagged protein was expressed in bacteria and purified using standard protocols.

GST pull-down

In vitro translations were performed using the TNT system (Pharmacia) and [³⁵S]-methionine (NEN). For experiments in figure 1, 1 μl of *in vitro* translation product was mixed with 250 μl of GST or GST-SF1f extract corresponding to 5 ml of the BL21 cells cultures, in GSB buffer (25 mM Hepes pH7.5, 100 mM KCl, 1 mM EDTA, 0.1% NP40 and 10% glycerol) with 1 mM DTT and antiprotease mix from Roche. For experiments in figure 4, *in vitro* translation products were incubated with 1 μg purified GST or GST-SF1 in the presence of 1 μg BSA as a non specific competitor. After 90 min incubation at 4 °C, 10 μl of glutathione beads (Pharmacia) were added for a further 30 min, beads were washed rapidly 5 times with GSB buffer, and proteins were analysed by SDS-PAGE, Coomassie blue staining and phosphorimaging (Cyclone, Packard).

Phosphorylation reactions

Phosphorylation reactions were performed as described previously [26]. Briefly, 20 μl reactions contained approximately 20 ng recombinant GST-KIS and 1 μg of substrate in 50 mM MES pH 8.0, 10 mM MgCl₂, 2 mM DTT, 2 mM EDTA, 25% glycerol, 10 μM [³²P]ATP (5 nCi/pmole) (NEN). For substrate and kinase mutant comparison we performed 30 minute reactions and checked the linearity of phosphate incorporation. For stoichiometric phosphorylation we performed 6 hour incubations with 0.4 mM ATP.

Mass spectrometry

After phosphorylation by KIS, SF1f was dialysed against NH₃HCO₃ 50 mM pH 8.0, digested with trypsin, and HPLC reverse phase fractions were analysed with a FT-ICR mass spectrometer APEX III (Bruker Daltonics, Bremen, Germany) equipped with a 7 Tesla supraconducting magnet and an infinity cell.

Metabolic labelling of cells

HEK293 cells were transfected using lipofectamine reagent (Invitrogen). After 24 hours, the medium was replaced with 1 ml methionine-free medium containing 1 mCi [³²P]-inorganic phosphate (NEN) for 4 hours. Cells were lysed in immunoprecipitation buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 1% nonidet P40, 1 mM EDTA, 1 mM DTT and protease inhibitors. Immunoprecipitation was performed with the monoclonal anti-myc 9E10 antibody (Santa Cruz Biotechnology).

Phosphopeptide mapping

Proteins were digested 'in-gel' overnight with trypsin at 30°C as previously described [26]. Eluted peptides were lyophilized and resuspended in electrophoresis buffer (pH 3.5). After electrophoresis and chromatography, plates were revealed by phosphorimaging.

Phosphatase treatment

Cell extracts (5µg of proteins) were treated with the indicated amount of Calf Intestine Phosphatase (CIP) (New England Biolabs) for 2 hours.

RNA gel-shift assays

RNA oligonucleotide with sequence derived from the Adenovirus major late pre-mRNA (5'-UUCGUGCUGACCCUGUCCCUUUUUUUUCCACAGC-3') was synthesized by Dharmacon. 5' labelling was performed with [γ -³²P]ATP and T4 polynucleotide kinase. Labelled RNA was purified on Nensorb20 column (NEN). About 10000 cpm/2 fmole of oligonucleotide was used with the indicated concentrations of purified proteins and with tRNA (0.5µgµl) as non specific competitor. Interactions were in 15µl containing 25 mM Tris pH7.5, 90 mM NaCl and 1 mM EDTA, for 1 hour at room temperature. We checked that equilibrium was reached at this time point. The mixture was loaded on 6% acrylamide gels in 0.5xTBE buffer, and run at 4°C for 3 hours.

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Abbreviations

GST, glutathione-S-transferase; DTT, dithiothreitol; Hepes, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid.

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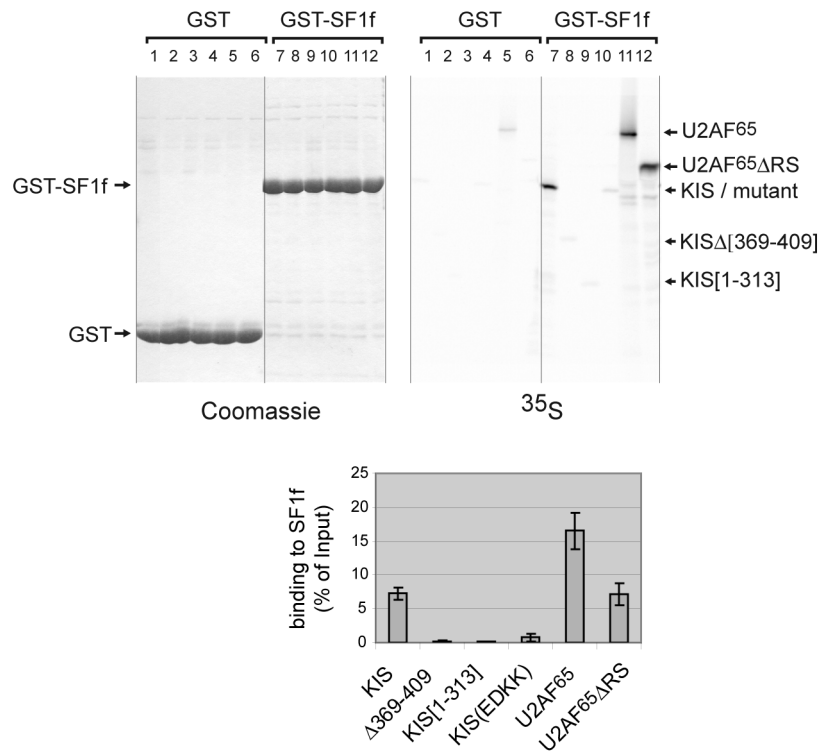


Figure 1. KIS interaction with SF1 *in vitro*

Various methionine labelled forms of KIS and U2AF⁶⁵ were tested for their binding to GST-SF1[1-255] (GST-SF1f) in a pull-down assay (top right, lanes 7-12). Lane 7: wildtype KIS; lane 8: KIS with a deletion within its UHM domain, KIS[Δ369-409]; lane 9: KIS lacking its UHM domain, KIS[1-313]; lane 10: KIS with mutations of E341 and D342 to lysine, KIS (EDKK), lane 11: full-length U2AF⁶⁵ and lane 12: U2AF⁶⁵ lacking its RS domain, U2AF⁶⁵ΔRS. Background binding on GST beads was analysed (lanes 1-6) and 0.5% of starting material was run in parallel to allow quantification (not shown). Mean values of three experiments are presented with standard deviations for binding to SF1f (bottom). The equivalent loading of the beads with GST and GST-SF1f was checked by Coomassie staining of the gel (top left).

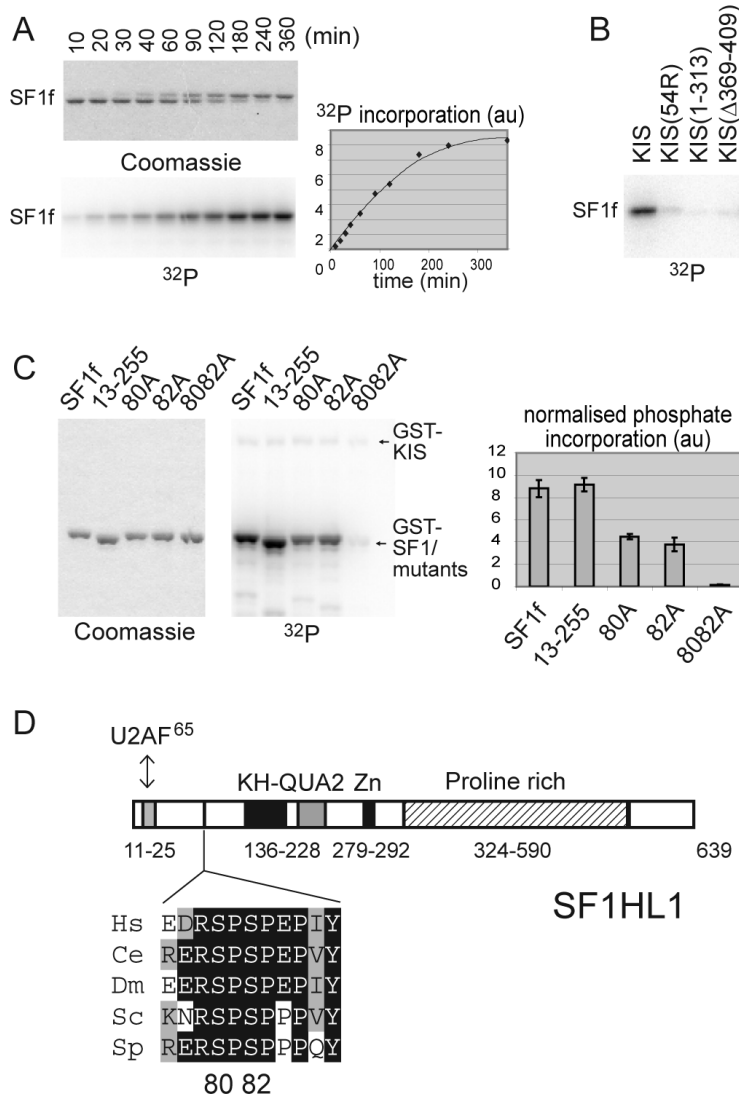


Figure 2. KIS phosphorylates SF1 *in vitro* on serines 80 and 82.

A) SF1[1-255] (SF1f) was phosphorylated by KIS with 10 μ M [γ -³²P]ATP for the indicated times. Reaction products were analysed by SDS PAGE, Coomassie blue staining and phosphorimaging. Phosphorylation of SF1f induced a shift of the SF1f band on SDS-PAGE.

B) The ability of similar amounts of the indicated forms of KIS to phosphorylate SF1f *in vitro* was compared, showing that deletion of or within the UHM domain of KIS impaired SF1f phosphorylation.

C) Phosphorylation *in vitro* of different forms of GST-SF1f as indicated. Reactions were stopped while phosphate incorporation was still linear with time (See “Experimental Procedures”). Mutation of both serines 80 and 82 completely abolished phosphate incorporation. Quantification: mean values of three experiments.

D) Primary structure of the SF1 isoform SF1HL1 (Alternatively spliced forms of SF1 differs by the length and sequence of their proline rich C-terminal region [28,51]). The SPSP motif is located in between the N-terminal U2AF⁶⁵ binding region and the KH-QUA2 domain that mediates recognition of the BPS [20,52]. The SPSP motif is in a highly phylogenetically conserved region as presented in the alignment [15]. The sequences of SF1 are from the

following organisms Hs, *Homo sapiens*; Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*.

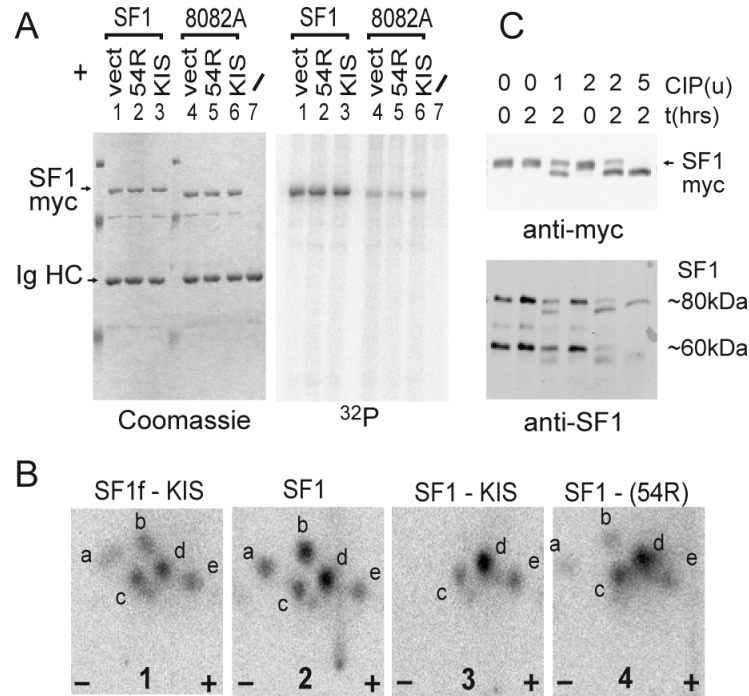


Figure 3. SF1 SPSP motif phosphorylation in cells.

A) HEK293 cells were transfected with SF1myc or SF1myc(8082A) together with either pCDNA3 (vect), pCDNA3-KIS(54R) (kinase defective) or pCDNA3-KIS. Overexpressed SF1myc was immunoprecipitated with antimyc mAb 9E10 and analysed by SDS PAGE, coomassie staining (left) and phosphorimaging (right).

B) Phosphorylated SF1 was analysed by tryptic phosphopeptide mapping. Map 1: SF1f phosphorylated by KIS *in vitro* (with a moderate stoichiometry of about 0.1 phosphate per SF1 molecule). Maps 2, 3 and 4 : phosphorylated SF1myc from lanes 1, 3 and 2 of gel in A. SF1f and SF1myc present almost identical maps showing that major phosphorylation occurs on the SPSP motif *in vivo*. Coexpression of KIS leads to disappearance of the more basic a and b peptides (two experiments) indicating an increase in the phosphorylation state of the SPSP motif.

C) 5µg of protein extract of HEK293 cells overexpressing SF1myc (top) or of untransfected HEK293 cells (bottom) were analysed by phosphatase (CIP) treatment with the different amounts indicated (units from New England Biolabs) and western blotting with antimyc or antiSF1 antibody (Cemines). In additional characterization experiments we showed that the 80kDa band migrated just below SF1myc, was nuclear and heat soluble which are characteristics of SF1 [53]. The 60kDa band most probably corresponds to a differentially spliced form [28].

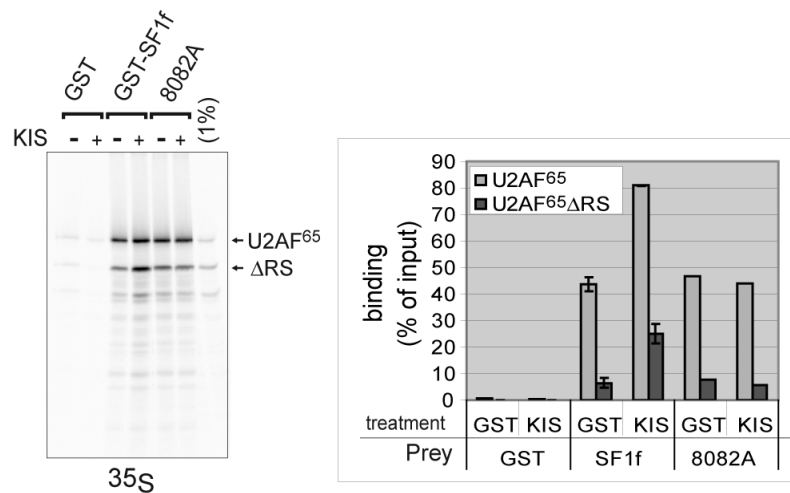


Figure 4. SF1 SPSP motif phosphorylation enhances binding to U2AF⁶⁵.

Pull-down experiments were performed using purified GST-SF1f or GSTSF1f-8082A that were phosphorylated by KIS (+) or mock phosphorylated (-). We used as input a mixture of in vitro translated methionine labelled U2AF⁶⁵ and U2AF⁶⁵ΔRS. We checked that interaction was the same when these proteins were alone or in the mixture (not shown). Interactions with GST-SF1f phosphorylated by KIS versus mockphosphorylated were in duplicates. Quantification was performed by phosphorimaging. The mean values with standard deviation are represented. Representative results of two experiments.

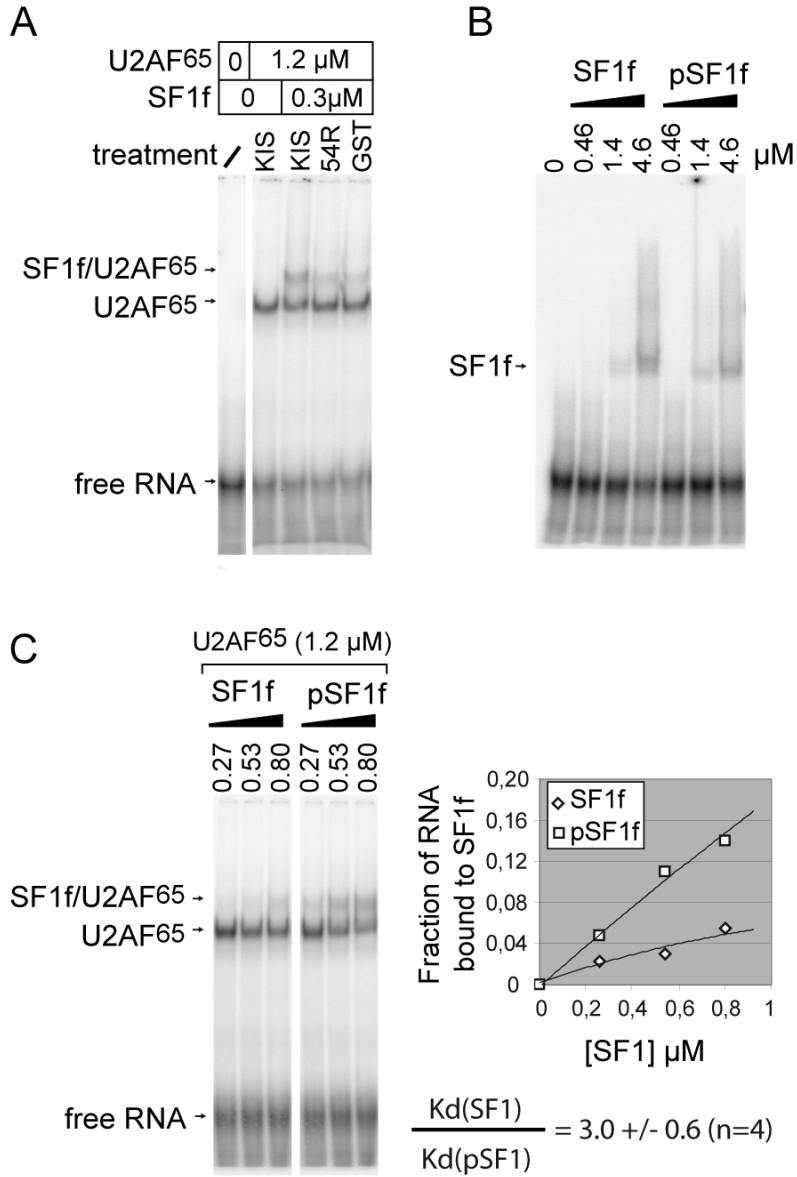


Figure 5. SF1 SPSP motif phosphorylation enhances formation of a SF1-U2AF⁶⁵-RNA ternary complex.

A. We used a ³²P labelled RNA oligonucleotide corresponding to a model 3' intronic sequence previously characterized in gel-shift experiments with SF1 and U2AF⁶⁵ (see Experimental Procedures) [14]. RNA was incubated with different protein mixtures. Single shift with U2AF⁶⁵ and supershift with U2AF⁶⁵ plus SF1f were clearly identified (lanes 2 and 3). Formation of SF1f-U2AF⁶⁵-RNA complex was enhanced by previous phosphorylation of SF1f by KIS (compare lane 3 with lanes 4 and 5).

B. Increasing concentrations of SF1f or SF1f phosphorylated by KIS (pSF1f) showed no major difference in RNA shift.

C. To quantify the enhancement of formation of the SF1f-U2AF⁶⁵-RNA complex upon phosphorylation of the SPSP motif, we used increasing concentrations of SF1f or SF1f phosphorylated by KIS (pSF1f) in the presence of 1.2 μM U2AF⁶⁵. The different bands were quantified by phosphorimaging and we plotted the formation of the ternary complex as a fraction of total RNA. We determined the decrease of the apparent K_d of SF1 for RNA upon

phosphorylation by KIS, by determining the y intercept of the Hill plot for each experiment (not shown). We used three different preparations of phosphorylated and mockphosphorylated SF1f in four experiments and found that phosphorylation induced an average 3 fold decrease of the apparent Kd of SF1 for RNA in the presence of 1, μ M U2AF⁶⁵.