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Phosphorylation–dephosphorylation differentially affects activities of splicing factor ASF/SF2

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SR proteins are a conserved family of splicing factors that function in both constitutive and activated splicing. We reported previously that phosphorylation of the SR protein ASF/SF2 enhances its interaction with the U1 snRNP-specific 70K protein and is required for the protein to function in splicing, while other studies have provided evidence that subsequent dephosphorylation can also be required for SR protein function, at least in constitutive splicing. We now show that the phosphorylation status of ASF/SF2 can differentially affect several properties of the protein. In keeping with a dynamic cycle of phosphorylation–dephosphorylation during splicing, ASF/SF2 phosphorylation was found to affect interaction with several putative protein targets in different ways: positively, negatively or not at all. Extending these results, we also show that, in contrast to constitutive splicing, dephosphorylation is not required for ASF/SF2 to function as a splicing activator. We discuss these results with respect to the differential protein–protein interactions that must occur during constitutive and activated splicing.

Keywords: phosphorylation status/splicing factor ASF/SF2/SR proteins

Introduction

Pre-mRNA splicing is catalyzed in a massive ribonucleoprotein complex called the spliceosome. Assembly of the U1, U2, U4/6 and U5 small nuclear ribonucleoproteins (snRNPs) into spliceosomes occurs in a sequential order (reviewed in Moore *et al*., 1993). U1 snRNP first recognizes the 5′ splice site to form the E (or commitment) complex, with base pairing between U1 snRNA and the 5′ splice site playing a critical role in recognition (reviewed in Madhani and Guthrie, 1994). U2 snRNP then binds the branch point sequence, forming the A complex, in an ATP-dependent manner that also involves base pairing (Madhani and Guthrie, 1994). Thirdly, U4/6 and U5 snRNPs together join the A complex to form a complex known as B1. In this complex, two important, major conformational changes occur: (i) 5′ splice site–U1 snRNA base pairing is disrupted (e.g. Konforti *et al*., 1993) and the 5′ splice site instead base-pairs with U6 snRNA; and (ii) base pairing between U4 and U6 snRNAs is disrupted and U6 becomes paired extensively with U2 snRNA. After these transitions, the spliceosome is referred to as the B2 complex, and becomes competent to catalyze the first step of splicing.

Spliceosome assembly also requires many non-snRNP protein factors (for reviews see Moore *et al*., 1993; Kramer, 1996). These include U2 auxillary factor (U2AF), which is required for A complex formation (Zamore *et al*., 1992), and members of the SR protein family, which are necessary for several steps in the splicing reaction (for review see Fu, 1995; Manley and Tacke, 1996; Valcárcel and Green, 1996). Each SR protein contains one or two N-terminal RNP-type RNA-binding domains (RBDs) and a C-terminal domain enriched in Arg–Ser dipeptide repeats. SR proteins initially were shown to function in pre-mRNA splicing as both general and alternative splicing factors. In the first case, they were found to complement splicing-deficient HeLa S100 extracts to allow splicing of a variety of pre-mRNAs (Krainer *et al*., 1990a, 1991; Ge *et al*., 1991; Fu and Maniatis, 1992; Zahler *et al*., 1992). In the second case, SR proteins were shown to modulate alternative splicing by affecting the usage of competing 5′ or 3′ splice sites in a concentration-dependent manner *in vitro* (e.g. Ge and Manley, 1990; Krainer *et al*., 1990b; Fu *et al*., 1992; Zahler *et al*., 1993), in transient transfections (e.g. Cáceres *et al*., 1994; Wang and Manley, 1995) and *in vivo* (Wang *et al*., 1998). SR proteins are also required for the activity of splicing enhancer elements, which are RNA sequences, most frequently found in exons, that are required to activate certain weak splice sites (e.g. Sun *et al*., 1993; Tian and Maniatis, 1993; Staknis and Reed, 1994; Ramchatesingh *et al*., 1995).

Studies of protein–protein and protein–RNA interactions have begun to provide insights into how SR proteins function in pre-mRNA splicing. The SR proteins ASF/SF2 and SC35 have been shown to interact with the U1 snRNP-specific 70K protein (U1 70K), and the RS domains of both proteins are required for this interaction (Wu and Maniatis, 1993; Kohtz *et al*., 1994). ASF/SF2 has also been shown to interact with the intact U1 snRNP (Kohtz *et al*., 1994; Xiao and Manley, 1997), and in addition is capable of binding 5′ splice sites (Zuo and Manley, 1994). These interactions are consistent with the observations that SR proteins increase the occupancy of U1 snRNP on 5′ splice sites (Eperon *et al*., 1993; Zahler and Roth, 1995) and that ASF/SF2 can cooperate with U1 snRNP to form a complex on the 5′ splice site (Kohtz *et al*., 1994; Jamison *et al*., 1995). In addition to U1 70K, SR proteins have been shown to interact with the 35 kDa subunit of U2AF, $U2AF^{35}$ (Wu and Maniatis, 1993). $U2AF^{35}$ interacts with its partner $U2AF^{65}$, which in turn binds to the pyrimidine tract near the 3′ splice site (Zamore *et al*., 1992). These protein–protein and protein–RNA interactions have the potential to bring the two splice sites together across either the intron (Wu and Maniatis, 1993) or the exon (reviewed by Berget, 1995). As splicing

activators, SR proteins bind specific sequences in RNA enhancers and form a complex with other proteins (e.g. Sun *et al*., 1993; Lynch and Maniatis, 1996; Zuo and Maniatis, 1996). This has been suggested to allow an interaction with U2AF^{35}, which can stabilize binding of the U2AF heterodimer to a poor pyrimidine tract to facilitate 3′ splice site recognition (Wang *et al*., 1995; Zuo and Maniatis, 1996).

SR proteins are phosphorylated extensively *in vivo* (e.g. Roth *et al*., 1990), and phosphorylation occurs mainly on serine residues within the RS domain (e.g. Colwill *et al*., 1996). A number of studies have demonstrated that protein phosphorylation and dephosphorylation are both required for splicing. Addition of the serine/threonine phosphatase PP1 to nuclear extract inhibits spliceosome assembly at a very early step (Mermoud *et al*., 1994), and phosphorylation of SR proteins is required for progression of the A complex to the B complex (Roscigno and Garcia-Blanco, 1995). Phosphorylation of ASF/SF2 has been shown to increase its interaction with U1 70K and U1 snRNP (Xiao and Manley, 1997), and to be required for splicing (Cao *et al*., 1997; Xiao and Manley, 1997). On the other hand, addition of phosphatase inhibitors (Mermoud *et al*., 1992; Tazi *et al*., 1992) to splicing reactions inhibits catalysis, but not spliceosome assembly, and thiophosphorylation of U1 70K (to prevent its dephosphorylation; Tazi *et al*., 1993) was shown to have a similar effect. Using thiophosphorylated ASF/SF2, Cao *et al.* (1997) provided evidence that dephosphorylation of ASF/SF2 can be required for splicing.

The above discussion indicates that ASF/SF2, and perhaps other SR proteins, probably undergo a cycle of phosphorylation–dephosphorylation during splicing. We therefore wished to investigate further the effects of phosphorylation on the activities of ASF/SF2. Contrary to its positive effect on interaction with 70K, phosphorylation of ASF/SF2 was found to affect interactions with other putative targets negatively, or not at all. Based on these differential responses to phosphorylation, we reasoned that different functions of ASF/SF2 might also display distinct phosphorylation requirements. Indeed, in contrast to its inactivity in constitutive splicing, thiophosphorylated ASF/SF2 was found to be able to activate enhancer-dependent splicing, suggesting that dephosphorylation is not required for ASF/SF2 to function as a splicing activator. We discuss these results in terms of a model that integrates the phosphorylation-dependent differences in protein–protein interactions with the differential requirements for dephosphorylation during constitutive and activated splicing.

Results

Differential effects of ASF/SF2 phosphorylation on protein–protein interactions

We previously reported that phosphorylation of ASF/SF2 enhances its binding to U1 70K and is necessary for splicing (Xiao and Manley, 1997). To extend these findings, we first examined whether phosphorylation affects interactions between ASF/SF2 and other putative target proteins. To this end, we used the glutathione *S*-transferase (GST) fusion protein interaction assay, as described previously (Xiao and Manley, 1997). We first examined the effect of ASF/SF2 phosphorylation on binding to itself and to another SR protein, Srp40. Yeast two-hybrid and far Western assays have provided evidence that SR proteins can interact (Wu and Maniatis, 1993), although the influence of phosphorylation on such interactions has not been investigated. GST–ASF/SF2 (GST–ASF) was expressed in and purified from *Escherichia coli*, phosphorylated or mock phosphorylated in HeLa S100 extract, repurified and used in binding assays as described in Materials and methods. *In vitro* translated, [^{35}S]methionine-labeled ASF/SF2 (Figure 1A) and Srp40 (Figure 1B) were incubated with phosphorylated (P) or mock-phosphorylated (M) GST–ASF, and bound proteins were analyzed by SDS–PAGE and autoradiography. The results allow two conclusions. First, mock-phosphorylated GST–ASF bound both proteins strongly, confirming that SR proteins can self-associate. [Similar results were obtained with untreated GST–ASF (data not shown). Binding was not mediated by RNA, since all assays were done in the presence of RNase.] Secondly, and most importantly, phosphorylation of GST–ASF greatly decreased binding to both proteins (Figure 1, compare lanes 3 and 4). The results do not reflect the use of S100 for phosphorylation, as identical results were obtained when a purified recombinant derivative of the SR protein kinase Clk/Sty (Colwill *et al*., 1996) was used to phosphorylate or mock-phosphorylate the proteins (results not shown; see also below). Together with our previous findings regarding U1 70K (Xiao and Manley, 1997), these results indicate that ASF/SF2 phosphorylation can have opposite effects on its interactions with different splicing factors.

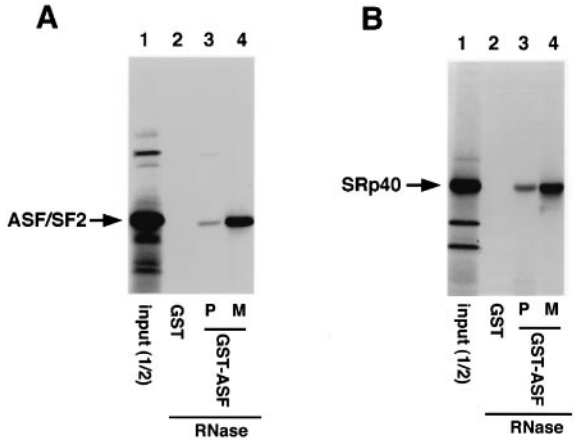

Fig. 1. Phosphorylation of GST–ASF decreases binding to SRp40 and ASF/SF2. Two micrograms of phosphorylated (P) and mock-phosphorylated (M) GST–ASF were bound to glutathione–agarose beads and used in binding reactions with *in vitro* translated, [^{35}S]methionine-labeled ASF/SF2 (**A**) or SRp40 (**B**). Samples were RNase treated, washed and the bound proteins were analyzed by 10% SDS–PAGE and fluorography. The positions of labeled SRp40 and ASF/SF2 are indicated.

We also examined the effect of phosphorylation on the ability of GST–ASF to interact with two other potential targets, a human homolog of the *Drosophila* splicing regulator Tra2, hTra2α and the small subunit of U2AF, U2AF^{35}. dTra2 and U2AF^{35} have both been shown previously to interact with ASF/SF2 (Wu and Maniatis, 1993; Amrein *et al*., 1994). Binding assays with phosphorylated and mock-phosphorylated GST–ASF and *in vitro* trans-

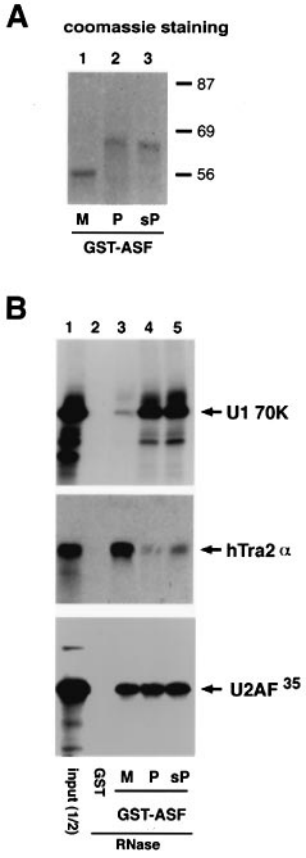

Fig. 2. Thiophosphorylation and phosphorylation of GST–ASF influence binding indistinguishably. Purified GST–ASF was thiophosphorylated with ATPγS (sP), phosphorylated with ATP (P) or mock-phosphorylated without ATP, using Clk/Sty kinase, and aliquots analyzed by SDS–PAGE and Coomassie Blue staining (**A**). Protein preparations were rebound to glutathione–agarose beads and used in binding assays with U1 70K, hTra2α and U2AF^{35}. Samples were treated with RNase, washed, and bound proteins were eluted and analyzed by 7 (U1 70K) or 10% (hTra2α and U2AF^{35}) SDS–PAGE and visualized by fluorography (**B**). The positions of U1 70K, hTra2α and U2AF^{35} are indicated.

lated proteins were performed as above, and the results are shown in Figure 2B (compare lanes 3 and 4; binding with U1 70K is shown for comparison). As observed with SR proteins, binding to hTra2α was greatly reduced by phosphorylation, perhaps reflecting the fact that Tra2 and SR proteins share related domain organization. Surprisingly, binding to U2AF^{35} was unaffected by phosphorylation. Our data thus indicate that phosphorylation can have diverse effects on ASF/SF2 protein interactions.

Phosphorylated and thiophosphorylated GST–ASF interact with other proteins indistinguishably

The above results raise the possibility that ASF/SF2 phosphorylation status might differentially affect the protein's activities in splicing, just as it can influence protein–protein interactions in distinct ways. As mentioned above, it has been shown that both phosphorylation (Xiao and Manley, 1997) and subsequent dephosphorylation (Cao *et al*., 1997) of ASF/SF2 can be necessary for splicing *in vitro*. Because the splicing reaction is extremely dynamic and complex, it is not feasible to follow the ASF/SF2 phosphorylation pattern through splicing and to correlate this with changes in specific protein–protein interactions. We therefore took another approach to address this question. Specifically, we set out to determine whether the same splicing-associated changes in the phosphorylation status of ASF/SF2 are required for all of the protein's activities. For example, it has been proposed that a key function of SR proteins in enhancer-dependent splicing activation is to facilitate U2AF binding via a direct interaction with U2AF^{35} (Zuo and Maniatis, 1996). Given our finding that the ASF/SF2–U2AF^{35} interaction is insensitive to the ASF/SF2 phosphorylation status, it is conceivable that the activity of ASF/SF2 in splicing activation might likewise be phosphorylation state insensitive. Although it is not possible to test the splicing activity of unphosphorylated ASF/SF2 (because the protein rapidly becomes phosphorylated under conditions that allow splicing), it is possible to analyze constitutively phosphorylated ASF/SF2, by use of thiophosphorylated ASF/SF2 (e.g. Tazi *et al*., 1993; Cao *et al*., 1997).

Before examining the ability of thiophosphorylated ASF/SF2 to participate in splicing activation, we wished to verify that thiophosphorylation affects protein–protein interactions in the same way as does phosphorylation. To test this, GST–ASF was thiophosphorylated with ATPγS (GST–ASF-sP) or phosphorylated with ATP (GST–ASF-P) and the modified proteins were repurified. Aliquots were analyzed by SDS–PAGE and Coomassie staining (Figure 2A), and the proteins were then used in binding assays with *in vitro* translated U1 70K, hTra2α and U2AF^{35}, using mock-phosphorylated GST–ASF as a control. The results (Figure 2B) show that thiophosphorylation (lanes 5) and phosphorylation (lanes 4) of ASF/SF2 had the same effects on interactions with all three proteins: in both cases, interaction with U1 70K was increased, with hTra2α decreased, and binding to U2AF^{35} was unaffected, when compared with mock-phosphorylated GST–ASF (Figure 2, lanes 3).

Thiophosphorylated ASF/SF2 cannot activate HIV tat pre-mRNA splicing in S100 extract

We first wished to verify that thiophosphorylated ASF/SF2 is inactive in splicing when its ability to function as a general splicing factor is required. As mentioned above, Cao *et al*. (1997) showed recently that thiophosphorylated ASF/SF2 could not activate splicing of an adenovirus-derived pre-mRNA (PIP7.A) in an S100 complementation assay. In our experiments, we chose first to analyze splicing of human immunodeficiency virus (HIV) tat pre-mRNA. Unlike PIP7.A, tat pre-mRNA is activated selectively by ASF/SF2 in nuclear extracts (Krainer *et al*., 1990a; Fu, 1993; Chandler *et al*., 1997). As discussed further below, this probably reflects the presence of an exonic splicing enhancer element(s) capable of responding *in vitro* to ASF/SF2. Tat pre-mRNA splicing can be activated by ASF/SF2 in S100 extract (Krainer *et al*., 1990a), probably reflecting the ability of the protein to function both as a general splicing factor and as a splicing activator. ASF/SF2 (histidine-tagged) was purified from *E.coli*, thiophosphorylated with ATPγS (ASF/SF2-sP) or phosphorylated with ATP (ASF/SF2-P), repurified, and aliquots analyzed by SDS–PAGE (Figure 3A). These proteins were then used in splicing assays with tat pre-mRNA and HeLa S100 extract. The results shown in Figure 3B indicate that thiophosphorylated ASF/SF2 was

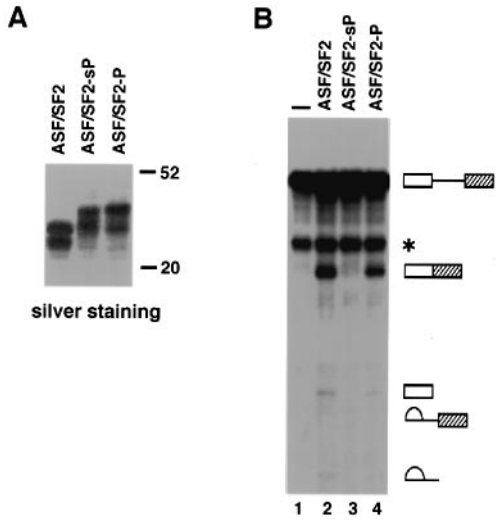

Fig. 3. Thiophosphorylated ASF/SF2 is unable to activate HIV tat pre-mRNA splicing in S100 extracts. (**A**) ASF/SF2 preparations. ASF/SF2 was phosphorylated with ATP (ASF-P), thiophosphorylated with ATPγS (ASF-sP) or mock-phosphorylated without ATP (ASF) using Clk/Sty kinase, and repurified. An aliquot of each preparation was resolved by 10% SDS–PAGE and detected by silver staining. (**B**) Splicing of tat pre-mRNA in S100 extract supplemented with ASF, ASF-P or ASF-sP. RNAs from splicing reactions were resolved on 6% polyacrylamide–8 M urea gels and visualized by autoradiography. Lane 1: splicing in the absence of added ASF/SF2; lanes 2–4: splicing in the presence of 0.8 μg ASF, ASF-sP or ASF-P, respectively. Indicated on the right are positions of (from top to bottom): pre-mRNA, mRNA product, 5′ exon, lariat intron–second exon intermediate and lariat intron product. A species resulting from cleavage unrelated to splicing is indicated by an asterisk (Krainer *et al*., 1990a).

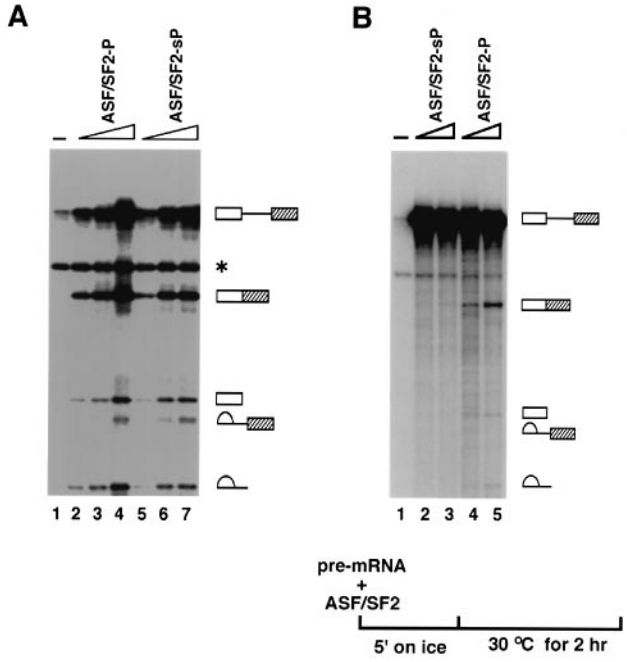

Fig. 4. Dephosphorylation of ASF/SF2 is differentially required for its roles in tat splicing. (**A**) Thiophosphorylated ASF/SF2 activates tat pre-mRNA splicing in nuclear extract. Tat pre-mRNA was incubated in reaction mixtures containing nuclear extract without added ASF/SF2 (lane 1), with 0.4, 0.6 or 0.8 μg of ASF-P (lanes 2–4) and with 0.4, 0.6 or 0.8 μg of ASF-sP (lanes 5–7). RNAs were recovered from the splicing reactions and analyzed as in Figure 3B. Symbols depicting the splicing substrate, products and intermediates are shown on the right as in Figure 3B. (**B**) Pre-incubation of ASF-sP, but not ASF-P, with tat pre-mRNA prevents splicing in nuclear extract. Either no SR proteins (lane 1), 0.4 and 0.6 μg of ASF-sP (lanes 2 and 3) or 0.4 and 0.6 μg of ASF-P (lanes 4 and 5) were pre-incubated with pre-mRNA on ice for 5 min, followed by addition of nuclear extract and incubation at 30°C for another 2 h, as indicated at the bottom of the figure. RNAs were analyzed as above.

unable to activate tat pre-mRNA splicing (Figure 3B, lane 3), while the mock-phosphorylated (lane 2) and phosphorylated (lane 4) proteins were both active. These findings indicate that, as observed with PIP7.A, thiophosphorylated ASF/SF2 is unable to function in at least one step during splicing of tat pre-mRNA.

Thiophosphorylated ASF/SF2 can function as a splicing activator

We next wished to determine whether dephosphorylation is required for ASF/SF2 to function as a splicing activator. HIV tat pre-mRNA has a weak 3′ splice site, reflecting both a poor branch site sequence and a poor polypyrimidine tract (Staffa and Cochrane, 1994; Si *et al*., 1997). Perhaps because of this, tat pre-mRNA splicing in HeLa nuclear extract is very weak, or undetectable, although it can be activated by addition of ASF/SF2 (e.g. Krainer *et al*., 1990a). Several purine-rich sequences in tat pre-mRNA exons resemble the ASF/SF2 recognition sequence defined by SELEX experiments (Tacke and Manley, 1995), and transfection experiments suggest that these may have splicing enhancer activity (Staffa and Cochrane, 1995). Consistent with this, ASF/SF2, but not SC35, has been shown to be able to commit tat pre-mRNA to splicing in an *in vitro* assay (Fu, 1993). Therefore, activation of tat pre-mRNA splicing in nuclear extract by ASF/SF2 is likely to be due to an exonic enhancer that is recognized by ASF/SF2 but not by SC35. SR proteins in a typical nuclear extract can function as general splicing factors, but perhaps the ASF/SF2 concentration is not high enough to activate splicing of the tat pre-mRNA. Therefore, if dephosphorylation of ASF/SF2 is not required for splicing activator function, we predict that thiophosphorylated ASF/SF2 will activate tat splicing in nuclear extract. However, if dephosphorylation is necessary for ASF/SF2 to function in this manner, thiophosphorylated ASF/SF2 should be inactive in this assay, as it was in S100 complementation. To test this, we carried out an experiment using ASF/SF2-P and ASF/SF2-sP to complement nuclear extracts for tat pre-mRNA splicing. The results are shown in Figure 4A: phosphorylated (lanes 2–4) and thiophosphorylated (lanes 5–7) ASF/SF2 both activated tat splicing in nuclear extract, consistent with the notion that dephosphorylation of ASF/SF2 is not required for the protein to function as a splicing activator.

In the above experiment, ASF/SF2-sP was slightly less efficient than ASF/SF2-P in activation of tat splicing. A possible explanation for this is that in the reaction mixture containing ASF/SF2-sP and nuclear extract, some of the spliceosomes were assembled with ASF/SF2-sP functioning as a general splicing factor, perhaps in the 5′ splice site complex containing U1 snRNP, and hence were unable to carry out splicing. Kohtz *et al*. (1994) have shown that pre-incubation of ASF/SF2 with pre-mRNA enhanced 5′ splice site complex formation, and Fu (1993) showed that pre-incubation of tat pre-mRNA with

ASF/SF2 allowed splicing upon subsequent addition of a 5′ splice site competitor RNA that would otherwise inhibit the reaction. Thus it is conceivable that pre-incubation of ASF/SF2-sP with the pre-mRNA prior to addition of nuclear extract will allow more ASF/SF2-sP, relative to endogenous SR proteins, to assemble into 5′ splice site complexes. A prediction of this is that pre-incubation of ASF/SF2-sP with tat pre-mRNA would not allow splicing upon addition of nuclear extract, contrasting with the situation observed when all components were added simultaneously. To test this, we performed splicing assays in which tat pre-mRNA was pre-incubated with ASF/SF2-P or ASF/SF2-sP prior to addition of nuclear extract, and the results are shown in Figure 4B. Strikingly, while pre-incubation of ASF/SF2-P and pre-mRNA activated splicing (Figure 4B, lanes 4 and 5), pre-incubation of ASF/SF2-sP with pre-mRNA did not allow any splicing following addition of nuclear extract (lanes 2 and 3). This finding supports our hypothesis that the functions of ASF/SF2 in general and activated splicing are separable and display differential requirements for dephosphorylation.

To extend the above results, we modified the S100-based complementation assay to differentiate between the general and sequence-specific activating functions of SR proteins. Specifically, in the case of tat pre-mRNA, we suggest that any SR protein can provide the general function, but only a subset, perhaps limited to ASF/SF2, can provide the activation function. If dephosphorylation is indeed not required for activator function, then ASF/SF2-sP should be capable of activating tat splicing in S100 in the presence of another SR protein. The results of such an experiment employing SC35 as the second SR protein are shown in Figure 5A. As expected, SC35 alone was unable to activate tat splicing in S100 (Figure 5A, lanes 3 and 4), although it was able to activate human β-globin splicing (Figure 5B, lanes 3 and 4). As shown above, ASF/SF2-sP alone (Figure 5A, lanes 5 and 6) was also unable to activate tat splicing. However, in combination with SC35, ASF/SF2-sP efficiently activated tat splicing (Figure 5A, lanes 7 and 8). These results provide strong support for our conclusion that the functions of SR proteins in general and activated splicing can be distinct, with dephosphorylation required in the former but not the latter.

Thiophosphorylated ASF/SF2 activates Drosophila doublesex pre-mRNA splicing

As mentioned above, it is likely that the substrate-specific effect of ASF/SF2 on tat splicing reflects binding to a purine-rich enhancer element. However, the tat RNA sequences required for splicing activation *in vitro* have not been determined, and we therefore wished to extend our results to a substrate with a better characterized splicing enhancer. To this end, we utilized the well-studied *Drosophila* doublesex (dsx) pre-mRNA, specifically a derivative containing only a portion of the dsx enhancer sequence, designated the purine-rich element (PRE; Lynch and Maniatis, 1995). The PRE was shown to be capable of activating splicing in HeLa nuclear extracts in the absence of the regulatory proteins Tra and Tra2 when situated 100 bases downstream of the regulated intron (dsxPRE pre-mRNA), and also to bind ASF/SF2 *in vitro* (Lynch and Maniatis, 1996). The dsxPRE pre-mRNA

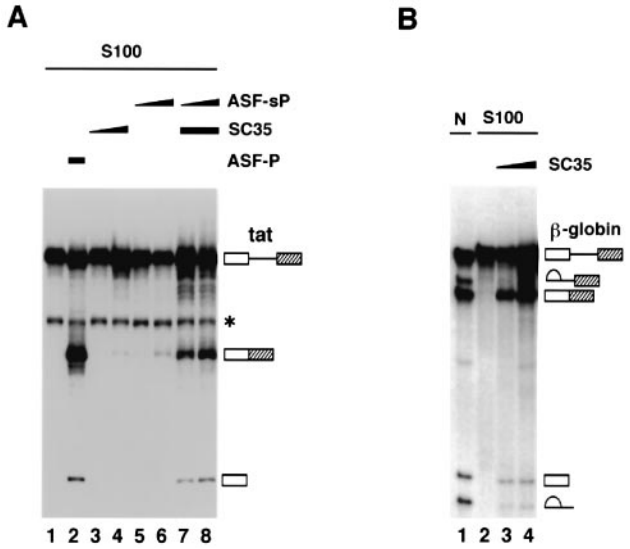

Fig. 5. The general and activation functions of SR proteins are separable. (**A**) ASF-sP together with SC35 can complement S100 extract for tat splicing. Splicing of tat pre-mRNA was carried out with 10 μl of S100 extract complemented with: no SR proteins (lane 1); 0.8 μg of ASF-P (lane 2); 50 (lane 3) and 100 ng (lane 4) of SC35; 0.6 (lane 5) and 0.8 μg (lane 6) of ASF-sP; 100 ng SC35 plus 0.6 (lane 7) and 0.8 μg (lane 8) of ASF-sP. Splicing reactions were carried out at 30°C for 2 h. RNA products were isolated and analyzed as in (B). (**B**) SC35 complements S100 extract for human β-globin pre-mRNA splicing. Splicing in either 5 μl of nuclear extract (N; lane 1) or 10 μl of S100 extract with 0 (lane 2), 50 (lane 3) or 100 ng (lane 4) of baculovirus-expressed SC35. RNAs from splicing reactions were analyzed on 6% denaturing polyacrylamide gels. On the right are symbols depicting the pre-mRNA, splicing products and intermediates.

could not be spliced in S100 extracts in the presence of ASF/SF2 or other SR proteins (results not shown), perhaps reflecting the previously observed requirement of some splicing enhancers for additional nuclear factors (Tacke and Manley, 1995; Tacke *et al*., 1997). We therefore employed a previously described assay (Yeakley *et al*., 1996; Tacke *et al*., 1998) in which an SR protein (in our case, ASF/SF2-P or ASF/SF2-sP) is added to a limiting amount of nuclear extract that by itself supports very little splicing, and the protein's ability to activate splicing determined. As shown in Figure 6, the dsxPRE pre-mRNA was spliced efficiently with 8 μl of nuclear extract (lane 1) but very poorly with 3 μl (lane 2). Not unexpectedly, splicing was strongly activated by addition of ASF/SF2-P (Figure 6, lanes 3 and 4; maximum ~8-fold). However, most importantly, ASF/SF2-sP also efficiently activated dsxPRE splicing (Figure 6, lanes 5 and 6; maximum ~5-fold), indicating that thiophosphorylated ASF/SF2 can activate enhancer-dependent splicing. Together, our results indicate that dephosphorylation is not required for ASF/SF2 to function as a splicing activator. Below we discuss the significance of these results and how they might reflect the varying effects of phosphorylation on different protein–protein interactions.

Discussion

Protein phosphorylation and dephosphorylation have been shown previously to be required for spliceosome assembly and activation of splicing catalysis, respectively. Our results show that the phosphorylation status of ASF/SF2

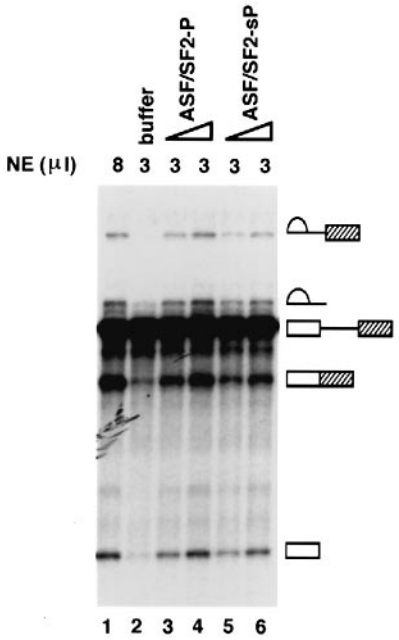

Fig. 6. Thiophosphorylated ASF/SF2 also activates *Drosophila* doublesex (dsxPRE) pre-mRNA splicing. Splicing reactions with dsxPRE pre-mRNA were carried out in reaction mixtures containing either 8 μl (lane 1) or limiting amounts (3 μl) of nuclear extract (lanes 2–6), supplemented with 0.4 and 0.6 μg of ASF/SF2-P (lanes 3 and 4) or 0.4 and 0.6 μg of ASF/SF2-sP (lanes 5 and 6). Reaction mixtures were incubated at 30°C for 1 h. RNAs were isolated and resolved in a 15% polyacrylamide–8 M urea gel. The symbols on the right depict (from top to bottom): lariat intron–second exon intermediate, lariat intron product, pre-mRNA, mRNA product and 5′ exon.

has distinct effects on the protein's interactions with various splicing factors. These results suggest the existence of dynamic protein–protein interactions during splicing that are modulated by phosphorylation and dephosphorylation. We also provide evidence that dephosphorylation is not required for ASF/SF2 to function as a splicing activator, although, as reported by Cao *et al*. (1997), it is required for the protein to participate in constitutive splicing. We discuss below how phosphorylation and dephosphorylation exert their roles in splicing by modulating protein–protein interactions, which suggests an explanation for why ASF/SF2 dephosphorylation is required for function in constitutive, but not activated splicing.

Dephosphorylation, specifically of ASF/SF2, has been shown to be required for splicing, although at a step subsequent to spliceosome assembly (Mermoud *et al*., 1992; Tazi *et al*., 1992; Cao *et al*., 1997). By what mechanism might this occur? We propose a model incorporating our current results with those of others to address this question, first discussing how dephosphorylation affects ASF/SF2's activities as a general splicing factor. Since inhibition of dephosphorylation using phosphatase inhibitors does not block spliceosome assembly, dephosphorylation must be required at a step after the spliceosome B complex is assembled. Since the B2 complex is active in catalysis (reviewed in Moore *et al*., 1993), the B complex assembled in the presence of phosphatase inhibitors must correspond to the B1 complex, and the transition from the B1 to B2 complex is therefore regulated, at least in part, by dephosphorylation. As discussed above, SR proteins are phosphorylated in the B1 complex. Upon dephosphorylation of ASF/SF2 and U1 70K, the interaction between ASF/SF and U1 snRNP will be weakened (Xiao and Manley, 1997) while, at the same time, interactions between SR proteins will be enhanced. These changes will shift the equilibrium away from formation of SR protein–U1 snRNP complexes to SR protein–SR protein complexes, helping, we suggest, U1 snRNP leave the 5′ splice site (e.g. Konforti *et al*., 1993) and allowing U6 snRNP to interact with it instead. It is likely that SR proteins participate directly in recruitment of the U4/U6/U5 snRNP complex to the 5′ splice site (Crispino *et al*., 1994; Crispino and Sharp, 1995; Roscigno and Garcia-Blanco, 1995; Tarn and Steitz, 1995), and an interesting possibility is that this reflects at least in part an interaction favored by SR protein dephosphorylation. In any case, inhibition of ASF/SF2 or U1 70K dephosphorylation has the potential to prevent this exchange of snRNPs and hence to block the spliceosome transition required for catalysis. This model is consistent with observations that thiophosphorylated U1 70K (Tazi *et al*., 1993) and ASF/SF2 (Cao *et al*., 1997) inhibit splicing catalysis but not spliceosome assembly.

We have provided evidence that, in contrast to its requirement for function in constitutive splicing, dephosphorylation is not required for ASF/SF2 to function as a splicing activator. In their role as activators, SR proteins have been shown to bind to RNA enhancers and, as one possible mechanism of activation, to stabilize binding of the U2AF heterodimer to weak polypyrimidine tracts, via a direct interaction with $U2AF^{35}$ (Wang *et al*., 1995; Lynch and Maniatis, 1996; Zuo and Maniatis, 1996). Since the phosphorylation status of ASF/SF2 has no significant effect on its interaction with $U2AF^{35}$ and, unlike the case of U1 snRNP, there is no evidence that U2AF leaves the polypyrimidine tract during splicing (Champion-Arnaud *et al*., 1995), there is no need for dephosphorylation for ASF/SF2 to function as a splicing activator by this mechanism. An alternative explanation is that the RS domain, the predominant site of phosphorylation, is dispensable for this function of ASF/SF2. However, this is probably not the case, because an RS domain deletion mutant of ASF/SF2 was unable to commit tat pre-mRNA to splicing (Chandler *et al*., 1997) and a similar mutant was unable to activate tat pre-mRNA splicing in our assay (unpublished data). Furthermore, recent studies have shown that an RS domain can in fact be sufficient to provide activator function when recruited to a pre-mRNA by a heterologous RNA-binding domain (Graveley and Maniatis, 1998).

What is the structural basis for the differential effects of ASF/SF2 phosphorylation on its interactions with other proteins? The most likely scenario is that they reflect differences primarily (or exclusively) in the RS domains of the interacting proteins. For example, the RS domains of SR proteins have repeating RS dipeptides, while the U1 70K and $U2AF^{35}$ RS domains have RD and RE, in addition to RS, dipeptides. SR proteins can be precipitated in the presence of 20 mM $MgCl_2$, a property used to purify SR proteins (Zahler *et al*., 1992), whereas U1 70K and $U2AF^{35}$ cannot. SR proteins are also recognized by monoclonal antibody (mAb) 104 (Roth *et al*., 1991), which recognizes a phosphorylated epitope in the RS domain of SR proteins, but not of U1 70K (Staknis and Reed, 1995).

Although hTra2α is different from classical SR proteins, it is recognized by mAb104 (Tacke *et al*., 1998). Furthermore, either RS domain of hTra2α, like those of other SR proteins, can substitute for the RS domain of ASF/SF2 in a cell viability assay, indicating that they perform similar functions (Wang *et al*., 1998). Additional studies will be required to understand the physical basis for these differential responses to ASF/SF2 phosphorylation.

Our observation that phosphorylation of ASF/SF2 decreases its binding to other SR proteins may also have implications for the mechanism by which the protein's subnuclear localization is determined. SR proteins have been shown to localize in the nucleus in structures known as speckles (reviewed in Misteli and Spector, 1997; Singer and Green, 1997). Speckles are probably storage sites of splicing factors, not the actual sites where splicing occurs. Instead, splicing appears to take place co-transcriptionally at sites removed from speckles (e.g. Zhang *et al*., 1994; Huang and Spector, 1996; Zeng *et al*., 1997). Several studies have provided evidence that phosphorylation–dephosphorylation controls the localization and subnuclear trafficking of SR proteins between speckles and sites of transcription/splicing (Misteli *et al*., 1997, and references therein). For example, addition of the SRPK1 kinase to permeabilized cells (Gui *et al*., 1994) or overexpression of the Clk/Sty kinase (Colwill *et al*., 1996) caused SR proteins to become dispersed throughout the nucleus. Using green fluorescent protein-tagged ASF/SF2 (GFP–ASF/SF2) in transfected living cells, Misteli *et al*. (1997) reported that GFP–ASF/SF2 localized to speckles, but displayed dynamic peripheral movement, especially when transcription was activated. Upon addition of a kinase inhibitor, speckles became rounded up while, in contrast, a phosphatase inhibitor caused the speckles to become less well defined, and GFP–ASF/SF2 dispersed in the nucleoplasm. Our observation that phosphorylation of ASF/SF2 decreased (or dephosphorylation enhanced) the protein's interaction with itself and other SR proteins provides a plausible mechanistic explanation of how phosphorylation and dephosphorylation can modulate SR protein localization. When dephosphorylated, the proteins would tend to self-associate, which could contribute to formation of the speckled structure, while phosphorylation would weaken such interactions, and hence facilitate dispersion of the speckles. The differential effects of phosphorylation on SR protein interactions thus have the potential to affect both splicing activity and subcellular trafficking.

Materials and methods

Expression and purification of recombinant ASF/SF2 proteins

Construction of plasmids encoding GST–ASF and His-ASF/SF2 has been described previously (Ge *et al*., 1991; Xiao and Manley, 1997). *Escherichia coli* cells (JM 101) were transformed with these plasmids and induced to express the fusion proteins with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 15°C overnight for GST fusions and at 37°C for 3 h for His-ASF/SF2. The recombinant proteins were affinity purified using glutathione–agarose beads for GST fusions and Ni^{2+}-agarose beads (involving denaturation and renaturation) for His-ASF/SF2 as described (Ge *et al*., 1991; Xiao and Manley, 1997). SC35 was expressed in recombinant baculovirus-infected cells and purified by ammonium sulfate fractionation and magnesium precipitation as described (Tacke *et al*., 1997).

Protein phosphorylation

His-tagged ASF/SF2-P and GST–ASF/SF2-P (100 μg) in 1.25 ml reaction mixtures were phosphorylated with ATP (or mock-phosphorylated without ATP) either with S100 extract under splicing conditions or with Clk/Sty kinase, repurified and dialyzed in buffer D as described (Xiao and Manley, 1997). His-tagged ASF/SF2-sP and GST–ASF/SF2-sP were prepared by thiophosphorylation of the recombinant proteins with 2 mM ATPγS (Sigma) and Clk/Sty kinase. For phosphorylation using Clk/Sty kinase, 50 μg of ASF/SF2 was incubated with 3 μg of GST–Clk/Sty kinase in phosphorylation buffer at 30°C for 1 h. The proteins were repurified and dialyzed in buffer D for GST–ASF or buffer D with 2.0 then 0.5 M guanidine chloride for ASF/SF2.

GST–protein interaction assay

GST-binding assays were carried out as described (Xiao and Manley, 1997). Briefly, 2 μg of phosphorylated, thiophosphorylated or mock-phosphorylated GST–ASF was allowed to bind to glutathione–agarose beads in binding buffer (NETN; 20 mM Tris pH 8.0, 100 mM NaCl, 0.5% NP-40 and 0.5 mM EDTA), and unbound proteins were washed away. *In vitro* translated, [^{35}S]methionine-labeled hTra2α, SRp40, ASF/SF2, U2AF^{35} or U1 70K (5 μl) were added to the beads in 200 μl of NETN. U2AF^{35} was translated in rabbit reticulocyte lysate acording to conditions described by Zhang *et al*. (1992). All the other proteins were produced in a TNT rabbit reticulocyte lysate (Promega). After binding, samples were treated with RNase and the beads were washed with NETN three times. Bound proteins were eluted by SDS sample buffer and analyzed by 10% SDS–PAGE and fluorography as described before (Xiao and Manley, 1997).

In vitro splicing

HIV tat pre-mRNA was prepared by *in vitro* transcription with SP6 RNA polymerase of pSP64-tat23 linearized with *Bam*HI (Krainer *et al*., 1990a; Xiao and Manley, 1997). The dsxPRE substrate was prepared by transcription of *Sma*I-linearized pdsxPRE by T7 RNA polymerase (Lynch and Maniatis, 1995). RNAs were purified from 6% polyacrylamide gels containing 8 M urea. Splicing reactions (25 μl) were carried out as described previously (Xiao and Manley, 1997) and contained 10 μl (or the indicated amounts plus buffer D) of nuclear extract or S100 extract and 5 μl of buffer D without glycerol. Both nuclear and S100 extracts were dialyzed in buffer D with 42 mM $(NH_4)_2SO_4$ instead of 100 mM KCl (Ge and Manley, 1990). Reaction mixtures contained 3.2 mM $MgCl_2$, 1 mM ATP, 20 mM creatine phosphate, 20 mM $(NH_4)_2SO_4$, 20 mM KCl, 2.6% polyvinyl alcohol, 0.5 mM dithiothreitol (DTT) and 4 U of RNasin (Promega), and were incubated at 30°C for 2 h for HIV tat or 1 h for dsxPRE. The products were treated with proteinase K, extracted with phenol/chloroform, precipitated with ethanol, and analyzed on 6 or 15% polyacrylamide gels containing 8 M urea for HIV tat and dsxPRE, respectively. Splicing reactions were repeated two to four times with entirely consistent results.

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