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The splicing factor-associated protein, p32, regulates RNA splicing by inhibiting ASF/SF2 RNA binding and phosphorylation

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The cellular protein p32 was isolated originally as a protein tightly associated with the essential splicing factor ASF/SF2 during its purification from HeLa cells. ASF/SF2 is a member of the SR family of splicing factors, which stimulate constitutive splicing and regulate alternative RNA splicing in a positive or negative fashion, depending on where on the pre-mRNA they bind. Here we present evidence that p32 interacts with ASF/SF2 and SRp30c, another member of the SR protein family. We further show that p32 inhibits ASF/SF2 function as both a splicing enhancer and splicing repressor protein by preventing stable ASF/SF2 interaction with RNA, but p32 does not block SRp30c function. ASF/SF2 is highly phosphorylated *in vivo*, a modification required for stable RNA binding and protein–protein interaction during spliceosome formation, and this phosphorylation, either through HeLa nuclear extracts or through specific SR protein kinases, is inhibited by p32. Our results suggest that p32 functions as an ASF/SF2 inhibitory factor, regulating ASF/SF2 RNA binding and phosphorylation. These findings place p32 into a new group of proteins that control RNA splicing by sequestering an essential RNA splicing factor into an inhibitory complex.

Keywords: ASF/SF2/p32/RNA splicing/SR proteins/SRp30c

Introduction

Removal of introns from pre-mRNAs is a crucial step in the process of eukaryotic gene expression. The temporal, developmental and tissue-specific regulation of pre-mRNA splicing has been established as an important control point of gene expression in metazoan cells.

Pre-mRNA splicing is a two-step enzymatic transesterification reaction, taking place in a large protein–RNA complex, the spliceosome. Assembly of the spliceosome occurs via a sequential recruitment of spliceosomal proteins and small nuclear ribonucleoproteins (snRNPs) to the pre-mRNA (reviewed in Moore *et al*., 1993). Much of the splicing regulation appears to occur at the early step of pre-mRNA recruitment to the spliceosomal assembly pathway. One of the crucial factors that partake in this initial step of pre-mRNA recognition is the SR family of splicing factors. SR proteins are a class of essential splicing factors (Manley and Tacke, 1996), with one or two N-terminal RNA-binding domains and characteristic arginine–serine (RS) di-peptide repeats of variable length in the C-terminus (Fu, 1995), hence the name SR proteins. A correctly 'built' early complex, or commitment complex, is then recognized by general splicing factors to build up a functional spliceosome that will perform the two transesterification reactions (reviewed in Moore *et al*., 1993). Conceptually, this type of complex assembly is analogous to pre-initiation complex formation in transcription.

The ASF/SF2 protein is a member of the SR family of splicing factors that can enhance constitutive RNA splicing and further has the capacity to regulate alternative splicing when overexpressed *in vivo* or added in excess to *in vitro* splicing assays (reviewed in Fu, 1995; Manley and Tacke, 1996). Even though the members of the SR family are highly homologous, they do not perform redundant functions, since an ASF/SF2 knockout in a chicken cell line cannot be complemented by SC35, another member of the SR family (Wang *et al*., 1996). SR proteins function as activators or repressors of splicing, depending on where they bind on the pre-mRNA (Kanopka *et al*., 1996). Typically, SR proteins enhance recognition of sub-optimal splice sites by binding to downstream exonic splicing enhancer elements. However, SR protein binding to an intron of the pre-mRNA can convert a splicing enhancer element to a splicing repressor element (Kanopka *et al*., 1996). In addition, SR proteins interact with one another and help to recruit other non-classical SR proteins to the spliceosome through RS domain interactions (Zuo and Maniatis, 1996). Other essential splicing factors, such as U1-70K and U2AF35, contain regions with RS repeats and have been shown to interact with SR proteins (reviewed in Fu, 1995). Importantly, the phosphorylation status of SR proteins can affect their function differentially in pre-mRNA recognition, spliceosome assembly and splicing catalysis (Cao *et al*., 1997; Xiao and Manley, 1997; Kanopka *et al*., 1998).

The cellular p32 protein was isolated first as a protein tightly associated with ASF/SF2 purified from HeLa cells (Krainer *et al*., 1991). The original study did not show any direct splice site regulatory activity of p32 (Krainer *et al*., 1991). However, a possible significance of the p32–ASF/SF2 interaction in splicing was not excluded. Subsequent studies have shown that p32 interacts with many cellular and viral proteins, including the lamin B receptor (Simos and Georgatos, 1994), herpes simplex virus 1 Orf-P protein (Bruni and Roizman, 1996), the adenovirus polypeptide V (Matthews and Russell, 1998), Epstein–Barr virus EBNA I protein (Wang *et al*., 1997;

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Chen *et al*., 1998) and the human immunodeficiency virus (HIV) Rev (Luo *et al*., 1994; Tange *et al*., 1996) and HIV Tat (Yu *et al*., 1995) proteins. The association between p32 and HIV Rev is particularly interesting since it suggests a possible connection between p32 and splicing control. The interaction between p32 and HIV Rev has been proposed to de-commit the HIV pre-mRNA from the spliceosome assembly pathway, thereby promoting transport of incompletely spliced HIV RNA (Tange *et al*., 1996).

In this study, we have re-investigated the significance of the p32–ASF/SF2 interaction in splicing, the concept being that p32 may not be a general splicing factor but one that modulates ASF/SF2 function. Here we show that p32 interacts with ASF/SF2 and another member of the SR protein family, SRp30c. Importantly, p32 inactivates ASF/SF2 as a splicing repressor or activator protein, but p32 does not have an effect on SRp30c function. Furthermore, p32 inhibits ASF/SF2 phosphorylation, either by HeLa nuclear extracts or SR protein kinases, a modification that is required for ASF/SF2 function in initiation of spliceosome assembly and splicing catalysis (Cao *et al*., 1997; Xiao and Manley, 1997).

The general conclusion emerging from this study is that p32 acts as a regulatory factor inactivating ASF/SF2 function by sequestering ASF/SF2 into an inhibitory complex. The results suggest that p32 acts similarly to transcription regulatory factors such as IκB and retinoblastoma tumour suppressor protein (Rb), which regulate pre-initiation complex formation by sequestering the NF-κB and the E2F transcription factors into inactive complexes.

Results

p32 inactivates ASF/SF2 as a splicing repressor protein

We have shown previously that in nuclear extracts prepared from uninfected HeLa cells (HeLa-NE), ASF/SF2 and other SR proteins act as repressor proteins. They inhibit IIIa splicing by binding to the 3RE, a regulatory sequence located immediately upstream of the IIIa branch site (Kanopka *et al*., 1996). As shown in Figure 1A, addition of increasing amounts of a recombinant p32 protein to HeLa-NE resulted in a concentration-dependent activation of IIIa splicing. The enhancement of IIIa splicing was specific, since addition of the same amount of p32 to splicing reactions performed with a β-globin pre-mRNA caused no activation (Figure 1B). To show that p32 reactivation of IIIa splicing was not due to simple ionic interactions between p32 and basically charged inhibitory SR proteins, we added a similar acidically charged protein, glucose oxidase (GOD), to the splicing assays. As shown in Figure 1C, GOD was not able to activate IIIa splicing.

To dissect further the sequence requirement for p32 activation of splicing, we tested the responsiveness of additional transcripts to p32. As summarized in Figure 1D, substituting the 3RE element with a β-globin sequence, which does not bind SR proteins (Kanopka *et al*., 1996), resulted in a loss of p32 activation [IIIa(–3RE)]. Similarly, a β-globin and an adenovirus control transcript, the L1 52,55K pre-mRNA, were also not activated by p32. We have shown previously that ASF/SF2 consensus RNA-binding sites can substitute functionally for the 3RE as a IIIa repressor element (Kanopka *et al*., 1996). Replacing the β-globin sequence in IIIa(–3RE) with two consensus ASF/SF2-binding sites restored the activation potential of p32 [IIIa(2ASF)]. Moreover, placing the 3RE element within the β-globin intron inhibits β-globin splicing (Kanopka *et al*., 1996), and rendered the transcript susceptible to p32 protein activation [β-glob(+3RE)]. Collectively, these results suggest that p32 inactivates SR proteins as repressor proteins of splicing.

We have demonstrated that SR proteins block IIIa splicing by inhibiting A complex formation at the IIIa 3′ splice site, most likely by binding to the 3RE and sterically interfering with U2 snRNP recruitment to the IIIa 3′ splice site (Kanopka *et al*., 1996). Therefore, if p32 alleviates the repressive effect of SR proteins on IIIa splicing, p32 should be able to activate A complex formation on a IIIa substrate RNA. In agreement with this hypothesis, pre-incubation of p32 with HeLa-NE increased the efficiency of A complex formation (Figure 1E), a result that explains the increase in IIIa splicing (Figure 1A). Since β-globin, IIIa(–3RE) and 52,55K are spliced more efficiently, the activation by p32 may have been obscured. However, this is not the case; analysis of splicing products and intermediates in a kinetic splicing experiment did not show an activation at any stage (data not shown). This and the complex assembly assay indicate that p32 acts at an early stage of spliceosome assembly. Furthermore, as shown in Figure 1F, addition of a single SR protein, ASF/SF2, was sufficient to abolish A complex formation on a IIIa 3′ splice site substrate RNA (lane 2). Importantly, addition of increasing amounts of p32 recovered much of the complex formation (lanes 3–5), suggesting that p32 inactivates ASF/SF2 as a splicing repressor protein. This type of activation of spliceosome assembly was not seen when the 3RE element was replaced by the β-globin sequence (data not shown).

p32 inactivates SR proteins as splicing enhancer proteins

We have shown previously that the position of SR protein-binding sites in the pre-mRNA determines whether they function as enhancers or repressors of splicing (Kanopka *et al*., 1996). Thus, moving the 3RE from its intron position into the downstream exon converts the 3RE from a repressor into a classical downstream exon splicing enhancer (Kanopka *et al*., 1996). Therefore, if p32 inhibits SR protein interaction with RNA (see below), one would predict that p32 should inactivate SR proteins as enhancer proteins of splicing. Using a transcript in which the 3RE element is positioned as a second exon splicing enhancer in a minimal adenovirus L1 52,55K pre-mRNA [52,55K(+3RE); B.Yue and G.Akusjärvi, in preparation], we show that p32 can inhibit the A-complex formation in HeLa-NE (Figure 1G). The wild-type 52,55K transcript lacking a characterized splicing enhancer was not inhibited by p32 (data not shown). The p32-induced activation of IIIa and inhibition of 52,55K(+3RE) A-complex formation confirm the notion that p32 functions as an ASF/SF2 inhibitory factor controlling the classical splicing regulatory activity of ASF/SF2.

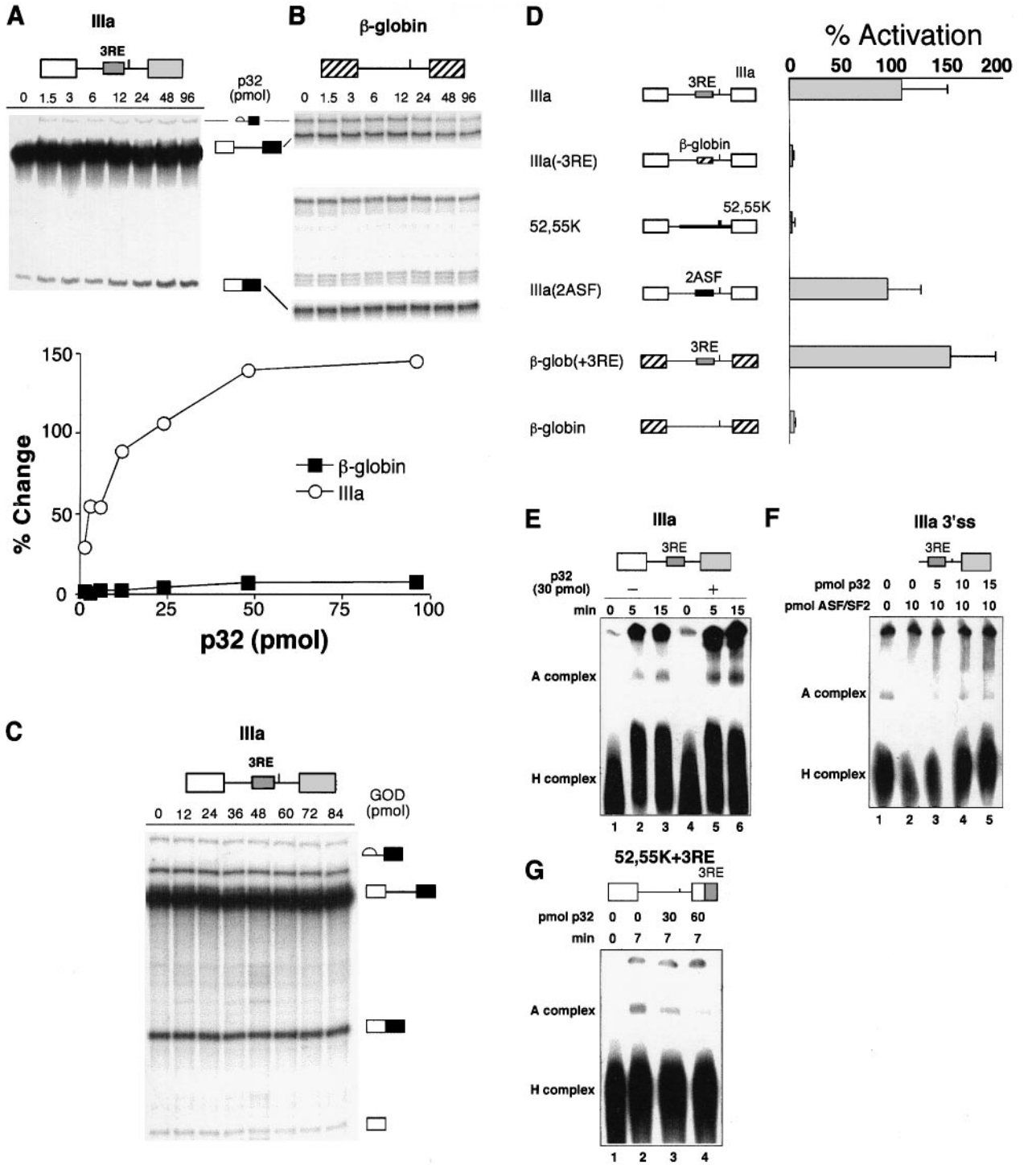

Fig. 1. p32 regulates splicing *in vitro*. (**A**) The indicated amounts of recombinant p32 were pre-incubated with HeLa-NE for 5 min at room temperature prior to the addition of the ^{32}P-labelled IIIa transcript. After 2 h, RNA was purified, and products were resolved by polyacrylamide gel electrophoresis and visualized by autoradiography. (**B**) As (A), except that splicing was programmed with a β-globin transcript. Lower panel: products were quantitated by PhosphorImager scanning; % Change refers to the change in splicing compared with no addition of p32. (**C**) GOD does not influence IIIa splicing. The indicated amounts of GOD were pre-incubated with HeLa-NE for 5 min before addition of IIIa RNA substrate. Products were resolved and analysed as in (A). (**D**) PhosphorImager quantitation of splicing using different transcripts as substrate. All transcripts were spliced three times (except IIIa, $n = 5$) in at least two different nuclear extract preparations. % Activation refers to the percentage change in splicing due to the addition of p32 protein as compared with no addition of p32. Error bars indicate standard deviations. (**E**) p32 enhances A complex formation on the IIIa pre-mRNA under splicing conditions. Samples were analysed for complex assembly at 0, 5 and 15 min intervals, either with or without pre-incubation of HeLa-NE and p32. (**F**) p32 alleviates ASF/SF2 inhibition of A complex formation on a IIIa 3′ splice site transcript. The indicated amounts of ASF/SF2 and p32 were pre-incubated at room temperature for 5 min followed by an incubation with HeLa-NE and RNA for an additional 20 min at 30°C. (**G**) p32 inhibits pre-spliceosome formation on a transcript with an exonic splicing enhancer. The 52,55K+3RE transcript was incubated under splicing conditions for 0 (lane 1) or 7 min (lanes 2–4), and analysed for complex assembly after pre-incubation of HeLa-NE with the indicated amounts of p32 for 5 min at room temperature.

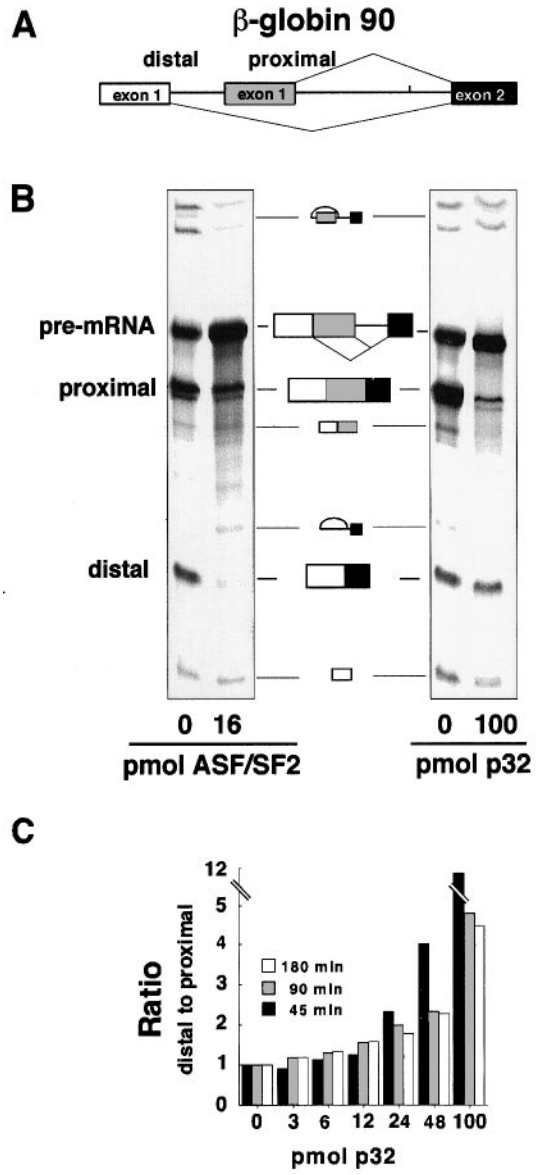

Fig. 2. p32 activates distal 5′ splice site usage in a chimeric β-globin substrate RNA. *In vitro* splicing conditions were as in Figure 1. (**A**) Schematic drawing of the β-globin 90 transcript (not drawn to scale); two alternative 5′ splice sites (exon 1) can be spliced to the 3′ splice site (exon 2). (**B**) The indicated amounts of p32 protein were pre-incubated with HeLa-NE for 5 min at room temperature before addition of substrate RNA. In the case of ASF/SF2, the protein was pre-incubated with the substrate RNA for 5 min at room temperature before addition of HeLa-NE. Reaction products were resolved by polyacrylamide electrophoresis and visualized by autoradiography. (**C**) Reactions containing different amounts of p32 protein were spliced for 45, 90 or 180 min. Splicing products were quantitated by PhosphorImager analysis. Ratio refers to the ratio of distal to proximal splice site product.

p32 regulates 5′ alternative splice site choice

One of the original functions attributed to SR proteins was their ability to regulate alternative RNA splicing (Ge and Manley, 1990; Krainer *et al*., 1990; Harper and Manley, 1991; Fu *et al*., 1992; reviewed in Fu, 1995). For example, using a β-globin model substrate RNA with duplicated 5′ splice sites, it was shown that ASF/SF2 caused a shift from distal to proximal 5′ splice site usage (Krainer *et al*., 1990). Since p32 inactivates SR proteins as splicing enhancer proteins (Figure 1G), one would predict that addition of p32 to an *in vitro* splicing reaction should have the completely opposite effect, i.e. a change from the proximal site to the distal site (Figure 2). In agreement with previously published results (Krainer *et al*., 1990), addition of ASF/SF2 to the *in vitro* splicing reaction caused a shift from the distal to the proximal site (Figure 2B, left panel). In contrast, addition of recombinant p32 caused a shift from proximal to distal 5′ splice site usage (Figure 2B, right panel). More detailed analysis of the splicing pattern, using different amounts of p32 in a kinetics experiment, demonstrated that p32 activated the distal 5′ splice site in a concentration-dependent manner (Figure 2C), and the effect of p32 was most pronounced at the earliest time point analysed (45 min), indicating that p32 acts at an early step of splicing. These experiments further demonstrate the antagonistic effects of p32 and ASF/SF2 in the regulation of splicing, and they also indicate that the effect of p32 is not restricted to a viral substrate transcript, but rather that p32 has a more general role in the regulation of splicing.

p32 relieves SR inhibition of IIIa splicing in transiently transfected HeLa cells

To demonstrate that p32 also functions as a regulator of SR proteins activity *in vivo*, we tested the effect of p32 on IIIa splicing in a transient DNA transfection assay. We used S1 analysis to detect quantitative differences in splicing activity. As shown in Figure 3A, co-transfection of low amounts of a plasmid expressing p32 activated IIIa 3′ splice site usage (left panel, lanes 2–3), whereas large amounts (lanes 4–5) caused an inhibition (see Discussion). Removal of the 3RE resulted, as previously shown (Kanopka *et al*., 1998), in a dramatic activation of IIIa splicing and, furthermore, annulled the stimulatory effect of p32 on IIIa splicing (right panel). Again, transfecting large quantities of the p32 plasmid caused an inhibition of mRNA accumulation in the cytoplasm (see Discussion). A PhosphorImager quantitation of three independent S1 analyses (average and standard deviation) is shown in Figure 3B. Although the experiment provides only indirect evidence linking p32 to splicing, it is in agreement with the hypothesis that the major effect of p32 on IIIa splicing *in vivo* works through an inactivation of SR proteins binding to the 3RE.

p32 blocks ASF/SF2 binding to RNA

Our previous data have shown that SR proteins, including ASF/SF2, suppress IIIa splicing by binding to the 3RE (Kanopka *et al*., 1996; data not shown). Since p32 does not bind RNA under splicing conditions (data not shown; Krainer *et al*., 1991), it seemed unlikely that p32 activated IIIa splicing by competing with ASF/SF2 for binding to the 3RE. It is more likely that p32 regulates ASF/SF2 function by affecting its RNA-binding capacity. To test this hypothesis, the effect of p32 on ASF/SF2 RNA binding was tested in a UV cross-linking assay. As shown in Figure 4A, addition of increasing amounts of recombinant p32 resulted in an inhibition of ASF/SF2 cross-linking to the RNA (lanes 2–4). To show that p32 is specific for ASF/SF2 and not just any RNA-binding protein, hnRNP A1 protein (half the amount of ASF/SF2) was included in the assay together with ASF/SF2. In the absence of the p32 protein, hnRNP A1 binding to the RNA substrate was not detected. However, subsequent to the addition of p32, which inhibited ASF/SF2

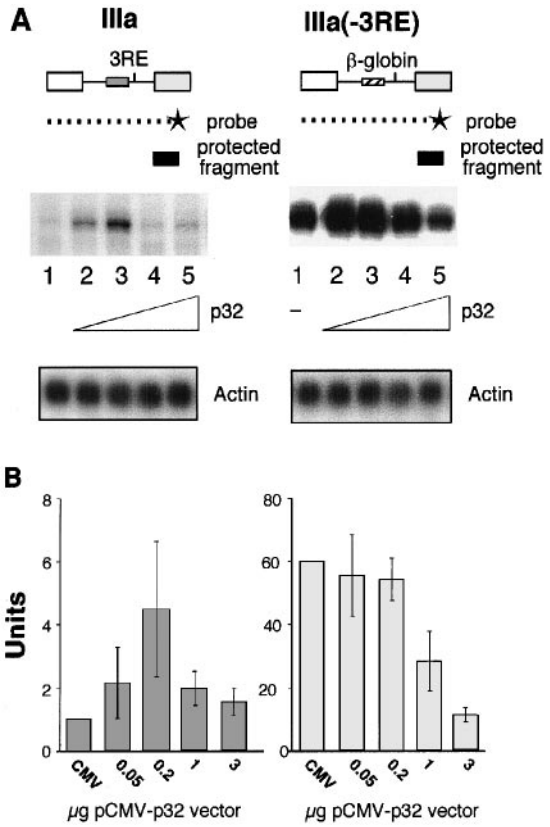

Fig. 3. p32 regulates IIIa splicing *in vivo* in transiently transfected HeLa cells. (**A**) Increasing amounts (lanes 2–5) of pCMV-p32 plasmid (0.05, 0.2, 1 or 3 μg) as well as an empty CMV control vector (lane 1) were co-transfected with constant amounts of reporter construct IIIa or IIIa(–3RE). Equal amounts of total cytoplasmic RNA were subjected to quantitative S1 analysis. Products were resolved by polyacrylamide gel electrophoresis and subjected to autoradiography (A) or PhosphorImager quantitation (B). The top panel in (A) shows a schematic representation of the pre-mRNAs expressed by the reporter constructs, with the 5′ end-labelled S1 probe shown below. The solid box denotes the fragment which is protected during the S1 analysis of the spliced products. Lower panel: a Northern blot probing for actin mRNA showing equivalent amounts of mRNA present in the S1 analysis. (**B**) PhosphorImager quantitation of the S1 analysis. All experiments were done at least three times, with average values and standard deviations shown.

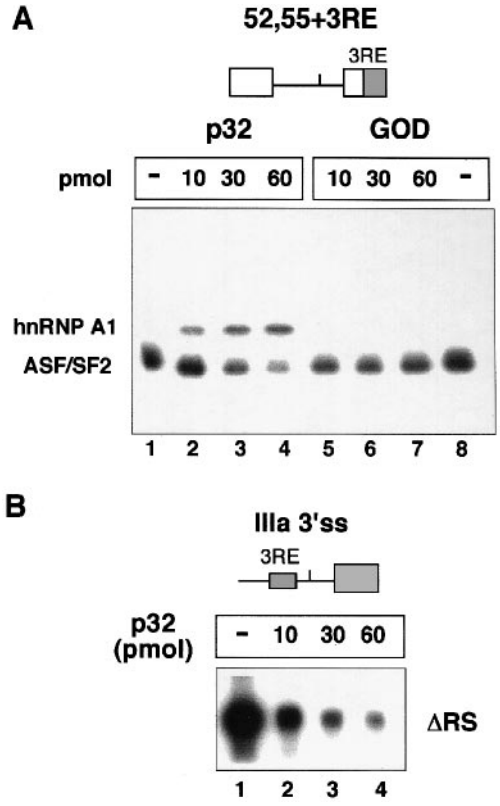

Fig. 4. p32 inhibits ASF/SF2 RNA binding. (**A**) Recombinant ASF/SF2 (20 pmol) and hnRNP A1 (10 pmol) were UV cross-linked to the 52,55K+3RE transcript (shown schematically at the top) in the absence (lanes 1 and 8) or presence of increasing amounts of recombinant p32 protein (10, 30 and 60 pmol, lanes 2–4, respectively) or GOD (10, 30 and 60 pmol, lanes 5–7, respectively). (**B**) Recombinant ΔRS (30 pmol) was UV cross-linked as described in (A), using the wild-type IIIa 3′ splice site transcript (Kanopka *et al*., 1996).

binding, hnRNP A1 was able to bind to the RNA. Also, the similarly charged protein GOD was unable to inhibit the RNA-binding capacity of ASF/SF2 (lanes 5–7) and, therefore, hnRNP A1 was not able to bind the RNA.

It has been speculated that the interaction of p32 with ASF/SF2 is due to ionic interactions between the highly basic RS domain in ASF/SF2 and the acidic C-terminus in p32 (Yu *et al*., 1995). Arguing against this hypothesis is the observation that GOD does not influence ASF/SF2 activity. Furthermore, to demonstrate that p32 interacts, at least in part, with the RNA-binding domain of ASF/SF2, we used a mutant ASF/SF2 protein lacking the RS domain (ΔRS) in a UV cross-linking assay. Similarly to the inhibition of the full-length ASF/SF2 protein (Figure 4A), the ΔRS protein was also inhibited in its RNA-binding capacity by p32 (Figure 4B), strengthening the conclusion that p32 regulates ASF/SF2 function at the level of a specific RNA-binding interaction.

p32 interacts with a second member of the SR protein family, SRp30c, without blocking its activity

Our results show that p32 functions as an ASF/SF2 inhibitory factor, functionally disabling its splicing regulatory activities. To determine whether p32 possessed the capacity to bind to other SR proteins, we tested the SRp30c protein. Previous studies have shown that SRp30c functions as a splicing activator in an S100 complementation assay (Screaton *et al*., 1995). We have also found that SRp30c, like ASF/SF2, inhibits IIIa splicing *in vivo*, in transiently transfected HeLa cells (C.Estmer, S.K.Petersen-Mahrt., J.Cáceres, A.Krainer and G.Akusjärvi, in preparation). In a far-Western analysis (Figure 5A), as expected (Krainer *et al*., 1991), p32 interacts with ASF/SF2 (Figure 5A, lane 1). Importantly, however, p32 also interacted with SRp30c in this type of assay (lane 2), albeit with a slightly lower efficiency compared with ASF/SF2 (lane 1 versus 2). Addition of SRp30c to *in vitro* splicing reactions demonstrated that this SR protein, like ASF/SF2 (Kanopka *et al*., 1996, and Figure 1), inhibits IIIa splicing (Figure 5B). Interestingly, addition of p32 to SRp30c did not rescue spliceosome assembly on the IIIa pre-mRNA (Figure 5C). Here, addition of ASF/SF2 or SRp30c blocks pre-spliceosome formation on the IIIa pre-mRNA (compare lane 1 with lanes 2 and 4). As is also shown above (Figure 1F), addition

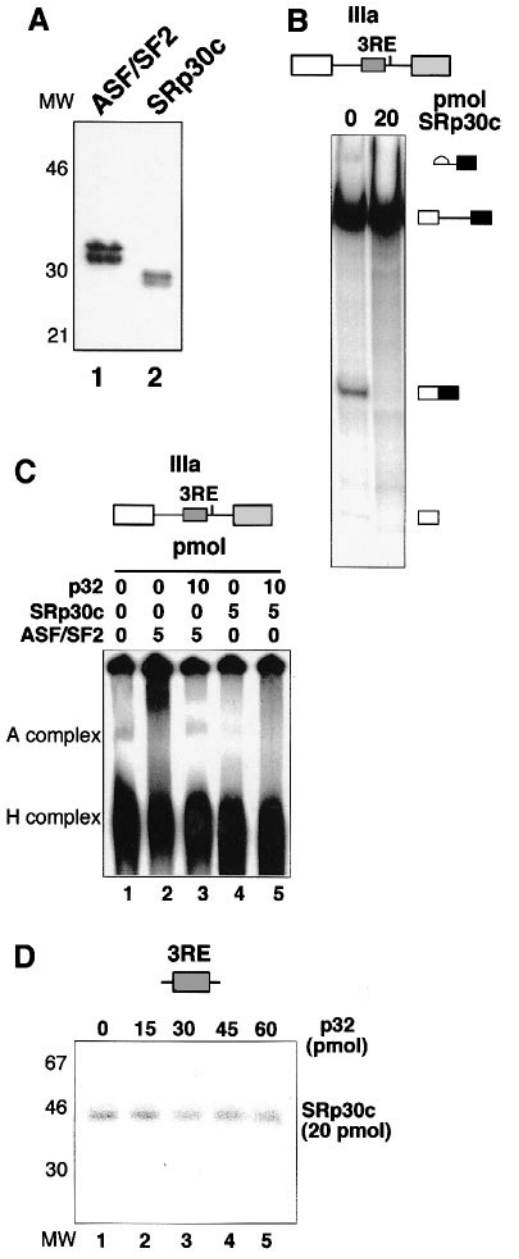

Fig. 5. p32 does not affect SRp30c function. (**A**) p32 can interact with SRp30c protein in a far-Western assay. Twenty picomoles of ASF/SF2 and SRp30c were detected as in Figure 6A. (**B**) SRp30c inhibits IIIa splicing. Splicing reactions were performed and analysed as in Figure 1A, with the 20 pmol of recombinant SRp30c added to the RNA substrate (IIIa) 5 min prior to addition of HeLa-NE. (**C**) p32 cannot alleviate SRp30c inactivation of IIIa A-complex formation. The indicated amounts of recombinant proteins were incubated with substrate RNA (IIIa) for 5 min prior to addition of HeLa-NE and complex analysis as in Figure 1E. (**D**) p32 does not inhibit the RNA-binding capacity of SRp30c. UV cross-linking was performed as in Figure 4A with 20 pmol of SRp30c and the indicated amounts of p32 protein. 3RE was used as substrate RNA.

of p32 abolishes the inhibitory effect of ASF/SF2 on spliceosome formation (lane 3). In contrast, p32 could not rescue SRp30c inhibition of IIIa spliceosome formation (lane 5). These findings suggest that p32 can interact with both ASF/SF2 and SRp30c, but can only modulate the activity of ASF/SF2. Further support for this specificity is provided by the observation that p32 could not block the RNA-binding capacity of SRp30c (Figure 5D). This

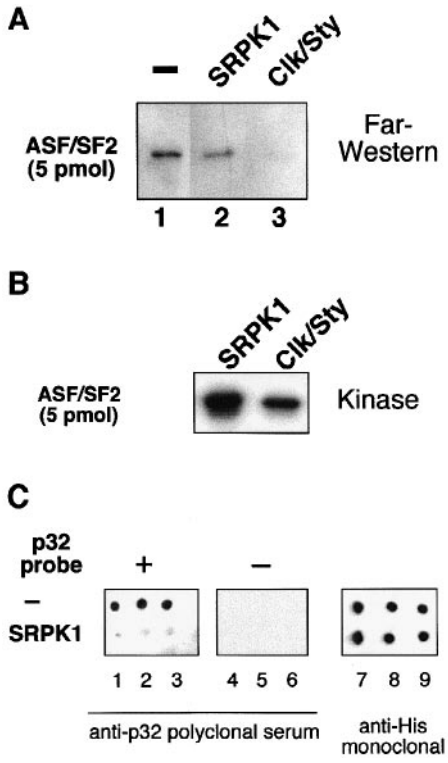

Fig. 6. p32 interacts preferentially with hypophosphorylated ASF/SF2. (**A**) Five picomoles of ASF/SF2 were subjected to *in vitro* phosphorylation in the absence (lane 1) or presence of SRPK1 (lane 2) or Clk/Sty (lane 3) protein kinases, and subsequently analysed by far-Western analysis, using 10 μg/ml of recombinant untagged p32 as a probe. (**B**) *In vitro* phosphorylation of 5 pmol of ASF/SF2 with SRPK1 or Clk/Sty for 15 min at room temperature, showing the difference in total phosphate incorporation. (**C**) Far-Western dot-blot. Two picomoles of ASF/SF2 were treated or not as in (A) and added directly to the filters. Subsequently, the filters were treated as in (A), with (lanes 1–3) or without (lanes 4–6) p32, followed by anti-p32 antiserum, or with anti-His antiserum (lanes 7–9). All experiments were done in triplicate.

is in contrast to ASF/SF2, where the same amount of p32 effectively inhibited RNA binding (Figure 4A). Taken together, our results suggest that p32 may show some specificity towards SR proteins, not disabling all SR proteins equally. Thus, binding of p32 to an SR protein does not necessarily block its activity.

The phosphorylated status of ASF/SF2 determines p32 binding

To analyse the direct interaction of ASF/SF2 and p32, far-Western protein–protein interaction studies were performed. As shown in Figure 6A, the interaction of p32 with recombinant ASF/SF2 is dependent on the status of ASF/SF2 phosphorylation. Pre-incubation of ASF/SF2 with the RS domain-specific SRPK1 protein kinase (Gui *et al*., 1994) weakly decreased the interaction of p32 and ASF/SF2 (compare lanes 1 and 2). A more dramatic effect was observed when the dual-specificity Clk/Sty kinase (Colwill *et al*., 1996), another SR kinase, was used (lane 3). Detecting filter-bound ASF/SF2 with an anti-His tag antibody demonstrated that equal amounts of proteins were loaded (data not shown). Importantly, ASF/SF2 is phosphorylated more strongly by SRPK1 than by Clk/Sty (Figure 6B), arguing against the notion that the number of phosphorylation sites determines p32 binding. More

probably, phosphorylation of specific sites in ASF/SF2 regulates p32–ASF/SF2 interaction. The inability of p32 to bind to Clk/Sty-phosphorylated ASF/SF2 is of particular interest since Clk/Sty has been suggested to regulate alternative RNA splicing through ASF/SF2 *in vivo* (Duncan *et al*., 1997).

The data presented in Figure 6A suggest that Clk/Sty phosphorylates an amino acid(s) that directly determines p32 interaction, whereas RS domain phosphorylation by SRPK1 does not significantly impair p32 interaction. However, protein–protein interactions depend on the correct folding of the proteins. Thus, we considered the possibility that the small effect of SRPK1 on p32–ASF/SF2 interaction (Figure 6A) was due to improper refolding of ASF/SF2 after the harsh treatment in SDS–PAGE and electroblotting. To test this hypothesis, we used a dot-blot far-Western approach (Figure 6C). Here, the SRPK1-treated or untreated ASF/SF2 was added directly to the nitrocellulose filter, keeping the protein in its 'native' phosphorylated form. The results clearly show that with equal amounts (lanes 7–9) but differentially treated ASF/SF2 (upper versus lower), p32 binds less efficiently to the SRPK1-phosphorylated ASF/SF2 (lanes 1–3). Omitting the recombinant p32 in the far-Western procedure did not produce a signal, demonstrating the specificity of the antiserum (lanes 4–6). We interpret these results to indicate that Clk/Sty phosphorylates a residue(s) that directly affects p32–ASF/SF2 interaction, whereas SRPK1-induced RS domain phosphorylation affects p32 interaction indirectly through ASF/SF2 protein conformation. Collectively, our results suggest that the activity of p32 as a splicing regulatory protein may be controlled by the phosphorylation of specific sites on the target protein.

p32 inhibits ASF/SF2 phosphorylation

Recent studies have shown that phosphorylation of ASF/SF2 is a required modification for its function in spliceosome assembly (Cao *et al*., 1997; Tacke *et al*., 1997; Xiao and Manley, 1997) and a regulator of its function in alternative RNA splicing (Kanopka *et al*., 1998). Since p32 interacts with ASF/SF2, it might also control the accessibility of ASF/SF2 to specific protein kinases. To test this hypothesis, we investigated whether p32 interaction with ASF/SF2 resulted in an alteration of ASF/SF2 phosphorylation. As shown in Figure 7A, pre-incubation of p32 with ASF/SF2 before addition of SRPK1 resulted in an inhibition of ASF/SF2 phosphorylation. This inhibition was even more pronounced when ASF/SF2 was phosphorylated in HeLa-NE (Figure 7B). Interestingly, using Clk/Sty as a protein kinase, p32 inhibition of ASF/SF2 phosphorylation was the most effective (Figure 7C). This is in agreement with our observation that Clk/Sty phosphorylation has a more severe effect on p32 interaction when compared with SRPK1 phosphorylation (Figure 6A). Thus, amino acids phosphorylated by Clk/Sty are most significant for p32–ASF/SF2 interaction. The ΔRS protein, lacking the ASF/SF2 RS domain, is phosphorylated by HeLa-NE protein kinases. Again, p32 was able, albeit with a lower efficiency, to inhibit this phosphorylation (Figure 7D). Coomasie Blue staining of all the proteins demonstrated that the amounts were constant and not subject to proteolysis (data not shown). These results indicate that p32 not only regulates RS domain phosphorylation, but also controls the phosphorylation of other, potentially important sites in ASF/SF2. Preliminary data suggest that p32 inhibits ASF/SF2 phosphorylation by binding to the substrate rather than to the kinase (data not shown). In summary, these results suggest that p32 interaction with ASF/SF2 is dependent on its state of phosphorylation, and that p32 can regulate ASF/SF2 function by inhibiting its phosphorylation, and this in turn controls the function of ASF/SF2 for RNA binding and spliceosome assembly during general and alternative RNA splicing.

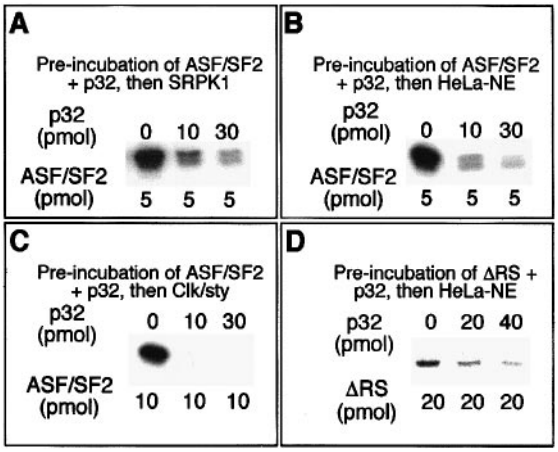

Fig. 7. p32 inhibits phosphorylation of ASF/SF2 and ΔRS. The indicated amounts of ASF/SF2 or ΔRS proteins were pre-incubated with increasing amounts of p32, followed by *in vitro* phosphorylation with SRPK1 (**A**), Clk/Sty (**C**) or HeLa-NE (**B** and **D**).

Discussion

The general conclusion emerging from our study is that the cellular p32 protein functions as an ASF/SF2 inhibitory factor, with a capacity to inactivate ASF/SF2 as a splicing repressor or splicing enhancer protein. Thus, p32 interaction with ASF/SF2 appears to have two important consequences for ASF/SF2 function. First, p32 inhibits the RNA-binding capacity of ASF/SF2 (Figure 4A) and, hence, its function in initiation of pre-spliceosome formation (Figure 1E). Secondly, p32 interaction with ASF/SF2 blocks ASF/SF2 phosphorylation (Figure 7), a post-translational modification which is required for ASF/SF2-mediated protein–protein interaction during spliceosome assembly (Xiao and Manley, 1997). Collectively, our results suggest that p32 may act as a new kind of splicing regulator that adjusts the activity of the essential splicing factor ASF/SF2 via changes in its subunit composition. Analogously to Rb, which binds and inactivates the E2F family of transcription factors, or IκB which inactivates NF-κB (Nevins, 1992; Baldwin, 1996; Whiteside and Israel, 1997), p32 may function as a modulator protein altering ASF/SF2 activity by sequestering it into an inactive complex.

It is important to note that, although we have used primarily model substrate RNAs based on the adenovirus L1 unit (Imperiale *et al*., 1995), we believe that our findings are of general significance for the control of RNA splicing in mammalian cells and not virus specific, since all experiments have been performed in NE prepared from

uninfected HeLa cells and the β-globin 5′ alternative splice site substrate was also regulated by p32 (Figure 2). The recent finding by Matthews and Russell (1998) that p32 interacts with adenovirus protein V suggests a probable function of p32 during lytic virus growth. We have initiated experiments aimed at further characterizing how adenovirus uses p32 for its own benefit.

The most direct mechanism by which p32 appears to influence ASF/SF2 activity is by inhibition of its RNA-binding capacity (Figure 4), an activity that cannot be mimicked by an equally acidically charged protein, GOD. We also show that p32 inhibits the RNA-binding capacity of an ASF/SF2 protein lacking the positively charged RS domain (Figure 4B). This finding indicates that the binding of p32 to ASF/SF2 is not simply a function of charge interaction with the RS domain. Supporting this idea is the observation that p32 was able to bind SRp30c (Figure 5A), but not able to inhibit SRp30c RNA binding or function (Figure 5).

The observed trade-off in binding between ASF/SF2 and hnRNP A1 (Figure 4A) is especially interesting, since a number of alternative splicing events can be regulated through the competition of these two proteins (Mayeda and Krainer, 1992; Mayeda *et al*., 1993; Sun *et al*., 1993; Yang *et al*., 1994; Chabot *et al*., 1997; Hanamura *et al*., 1998; Jiang *et al*., 1998). It has been hypothesized (Hanamura *et al*., 1998) that the differences in the molar amounts of ASF/SF2 and hnRNP A1 in various cell types is a key factor in regulation of alternative RNA splicing. Our results suggest that p32 may modulate this regulation further (Figure 4A), by chelating the ASF/SF2 component.

It should be noted that the effect of p32 on transcripts is, in some instances, not the most dramatic. However, importantly for those transcripts which do have a specific requirement for the ASF/SF2 protein, p32 regulation is most pronounced. The most simplistic explanation for this is that not all SR proteins are inactivated efficiently by p32. Such a conclusion is in line with our observation that p32 does not block SRp30c function, but can interact with SRp30c (Figure 5). Our preliminary data also suggest that p32 interacts with even more SR proteins and, at least in one case, also disables its function (data not shown).

The inhibitory effect of p32 on ASF/SF2 phosphorylation is likely to have significant consequences for ASF/SF2 function *in vivo*. For example, this post-translational modification appears to regulate the subcellular localization (Caceres *et al*., 1998) and sub-organelle distribution (Misteli and Spector, 1997) of SR proteins. There are multiple examples of inhibitory proteins regulating transcription that control the subcellular localization of transcription factors (reviewed in Latchman, 1991). Similarly, p32 may participate in splicing by controlling the sub-organellar or sub-cellular localization of SR proteins. Cycles of phosphorylation and dephosphorylation reactions have been proposed to reorganize SR proteins from interchromatin granules to active sites of splicing (Misteli and Spector, 1997). Also, some members of the SR protein family, including ASF/SF2, have been shown to shuttle between the nucleus and cytoplasm (Caceres *et al*., 1998), and the phosphorylation status was shown to be important for this activity. Additionally, SR protein phosphorylation has been shown to be important for the RNA-binding

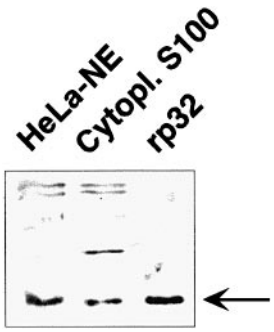

Fig. 8. Cytoplasmic and nuclear distribution of p32 in HeLa extracts. Western blot analysis of nuclear and cytoplasmic extracts using an anti-p32 polyclonal serum (Matthews and Russell, 1998). Lane 3 is a control showing the position of a recombinant p32 protein.

capacity of SR proteins (Tacke *et al*., 1997; Kanopka *et al*., 1998), the capacity of SR proteins to mediate protein–protein interaction (Xiao and Manley, 1997) and the induction of spliceosome assembly (Mermoud *et al*., 1994; Cao *et al*., 1997; Xiao and Manley, 1997). An effect of p32 on any of these activities would control the availability of ASF/SF2 for splice site regulation. However, the stage(s) at which p32 regulates splicing *in vivo* remains to be determined.

There is some controversy with regards to the subcellular localization of p32. It has been suggested to be present in the mitochondria (Muta *et al*., 1997; Matthews and Russell, 1998), cytoplasm (Luo *et al*., 1994; Simos and Georgatos, 1994; Tange *et al*., 1996; Wang *et al*., 1997), cell surface (Eggleton *et al*., 1995; Ghebrehiwet *et al*., 1997; Guo *et al*., 1997; Peterson *et al*., 1997) and nucleus (Luo *et al*., 1994; Simos and Georgatos, 1994; Tange *et al*., 1996; Wang *et al*., 1997; Matthews and Russell, 1998). Matthews and Russell (1998) found p32 associated both with the mitochondria and the nucleus, and hypothesized that the difference in location could be explained if p32 is a component of a transport system linking the nucleus to the mitochondria. The mitochondrial location of p32 in association with the complement component C1q has also been confirmed recently (Dedio *et al*., 1998). This is in contrast to the previous claim of cell surface location (Ghebrehiwet *et al*., 1997), a notion which has also been challanged by Muta *et al.* (1997). It is important, therefore, that in our studies we find a significant fraction of p32 present in HeLa-NE prepared by standard techniques (Figure 8). This finding should not be taken as strong support for the subcellular localization of p32. It is provided to show that p32 is present in the HeLa-NE and, therefore, cannot be disregarded as a regulatory factor in *in vitro* splicing experiments. The observation that ASF/SF2 appears to shuttle between the nucleus and the cytoplasm (Caceres *et al*., 1998) would be in accordance with the putative transport role of p32, and it may be that the activity of ASF/SF2 could be regulated by the relative binding affinity of p32 for other cellular (or viral) components. This could explain in part the discrepancy between the *in vivo* and *in vitro* effects of p32. *In vitro*, p32 did not have much of an effect on a reporter transcript lacking the 3RE element [IIIa(–3RE); Figure 1D], whereas *in vivo* p32 co-transfection inhibited IIIa(–3RE) expression at high concentrations (Figure 3). It is possible that large amounts of p32 protein sequester ASF/SF2 in the

cytoplasm, leaving a severe deficiency of ASF/SF2 in the nucleus. We are currently investigating the effect of p32 on the subcellular localization of SR proteins. The association of p32 with the ASF/SF2–Rev complex (Gilmartin *et al*., 1992; Yu *et al*., 1995; Tange *et al*., 1996) supports the notion that p32 may participate in RNA–protein shuttling from the nucleus to the cytoplasm. Alternatively, high amounts of p32 expression *in vivo* could cause a non-specific inhibition of RNA splicing.

With the potentially manifold activities of ASF/SF2 in cellular RNA metabolism, the association of p32 with ASF/SF2 before, during or after RNA transcription, splicing or transport are potentially key points for regulation. Viruses typically target key proteins in the cell and redirect their activity so that they will allow for efficient virus multiplication. Studies of virus replication have, therefore, contributed significantly to our understanding of important check points required for normal cell-cycle control. The best examples, so far, are the identification of the p53 tumour suppressor protein and the critical function of the Rb tumour suppressor protein in the control of the cell cycle. It is interesting, therefore, that a number of viruses appear to target the p32 protein. It may be that this provides another example of a 'hijacking' of a key cellular protein to support viral replication further. There is obviously still much more to learn about the function(s) of the p32 protein *in vivo*. This study sheds some light on its function by demonstrating that p32 has a capacity to regulate splicing by controlling the activity of an essential splicing factor, the ASF/SF2 protein.

Materials and methods

Plasmids, transcripts and recombinant proteins

Plasmid sequences are available on request or at http://www.bmc.uu.se/IMIM/res/GA.html.

Transcripts IIIa, IIIa(2ASF), IIIa(–3RE), β-globin, β-globin(+3RE), β-globin 90 (Reed and Maniatis, 1986) and 52,55K have been described previously (Kanopka *et al*., 1996, 1998). In transcript 52,55K+3RE, the 49 nucleotide long 3RE element (Kanopka *et al*., 1996) was appended as a second exon splicing enhancer six nucleotides downstream of the 52,55K 3′ splice site (B.Yue and G.Akusjärvi, in preparation). All pre-mRNAs were transcribed with T7 RNA polymerase from PCR-amplified products with a U1 snRNA-binding site at the 3′ end (Kreivi *et al*., 1991). His-tagged recombinant ASF/SF2 (Ge *et al*., 1991), ΔRS (kind gift of Jan-Peter Kreivi, previously used in Kanopka *et al*., 1996), Trx(thioredoxin-His)-SRp30c (kind gift from Jan-Peter Kreivi), hnRNP A1 (kind gift of Karin Öhman) and SRPK1 (Gui *et al*., 1994) were purified by standard Ni-column chromatography (Novagen). For some experiments (Figure 5A), the Trx tag of SRp30c was removed according to the manufacturer's protocol (Novagen). This changed the SDS–PAGE migration pattern from 43 to 30 kDa. Clk/Sty (Colwill *et al*., 1996) was expressed as a GST-tagged protein and purified as described in the Pharmacia–Amersham Biotech manual. Recombinant p32 was isolated as a His-tagged protein by standard Ni-column chromatography, or as an untagged protein by Mono-Q FPLC (Matthews and Russell, 1998). SDS–PAGE Coomasie analysis showed p32 to be >90% pure (data not shown). GOD was purchased from Sigma (St Louis, MO). All proteins were dialysed against buffer D (Dignam *et al*., 1983) containing 20% glycerol.

In vitro splicing reactions

Conditions for extract preparation and *in vitro* splicing were as described (Kreivi *et al*., 1991), except that the ATP concentration was raised to 2 mM, phosphocreatine (Sigma) was included at a concentration of 20 mM and the final $MgCl_2$ concentration was lowered to 2.8 mM [3.2 mM for β-globin 90 transcript (Reed and Maniatis, 1986)]. Reactions were incubated for 2 h at 30°C (or as indicated), proteins were digested with proteinase K, RNA was isolated and products were resolved on 8% PAGE–urea. S100 splicing reactions were performed as published (Krainer *et al*., 1990). PhosphorImager quantitation: percentage splicing = signal of product/(signal of product + signal of transcript)×100; percentage change = (percentage splicing of *x* – percentage splicing of 'zero' control)/percentage splicing of 'zero' control×100. All reactions were performed at least three times, and average and standard deviations are shown. Ratio [for β-globin 90] = (volume of distal spliced product)/(volume of proximal spliced product); arbitrarily setting the zero control lane to 1.

Pre-spliceosome formation

Conditions for pre-spliceosome formation are as for *in vitro* splicing. Using the indicated transcript, incubations were as follows. ASF/SF2 and p32 were pre-incubated at room temperature for 5 min, followed by addition of 15 μg of HeLa-NE and 20 fmol of ^{32}P-labelled transcript. Complex formation was monitored by electrophoresis on a 4% acrylamide:piperazine (84:1) gel and autoradiography (Kreivi and Akusjärvi, 1994).

Transfections and S1 analysis

Semiconfluent HeLa monolayer cells were transfected (three times) with 3 μg of reporter construct and 3 μg empty cytomegalovirus (CMV) vector or increasing amounts of pCMVp32 (0.05, 0.2, 1 and 3 μg) by the calcium phosphate co-precipitation technique (Ausubel *et al*., 1987) or by using the FuGene® cocktail (Boehringer Mannheim). At 16 h post-transfection, cells were glycerol shocked, and total cytoplasmic RNA isolated 30–32 h later. Equal amounts of RNA were subjected to S1 analysis (Ausubel *et al*., 1987) using the probe described by Kanopka *et al*. (1998). To ensure an equal amount of mRNA was used in the S1 analysis, a Northern blot with a human actin cDNA probe was performed (Ausubel *et al*., 1987).

UV cross-linking

Recombinant ASF/SF2, ΔRS, SRp30c and hnRNP A1 were incubated under splicing conditions with increasing amounts of p32 or GOD for 5 min at room temperature prior to addition of ^{32}P-labelled transcript. All reactions contained 50 ng of tRNA (Sigma) as a non-specific competitor. Mixtures were incubated for a further 5 min at 30°C, then cross-linked by UV irradiation (output: 1200 μW/cm^2, distance: 1 cm) for 10 min on ice. RNA was digested with 10 μg of RNase A (Pharmacia) at 37°C for 30 min. Products were resolved on 12 or 15% SDS–PAGE under reducing conditions and visualized by autoradiography.

Far-Western blotting

Recombinant SRp30c or ASF/SF2, with or without phosphorylation by SRPK1 or Clk/Sty, was subject to 12% SDS–PAGE under reducing conditions. Blotting was done under standard conditions (Burnette, 1981). Membranes were blocked in TBS-T-DM [5% dry milk (w/v), 50 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween-20], washed three times in TBS and 3 or 10 μg/ml of recombinant p32 was added in TBS-T-B [TBS-T with 1% bovine serum albumin (BSA)] for 30 min at room temperature (with and without 60 μg of GOD). They were then washed twice in TBS-T and rabbit polyclonal anti-p32 (Matthews and Russell, 1998) was added in TBS-T-B at a dilution of 1:500 for 12–16 h at 4°C (or 30 min at room temperature) prior to them being washed three times in TBS-T. Donkey anti-rabbit Ig–horseradish peroxidase (HRP) (Amersham) was added and incubated at 1:2000 dilution in TBS-T-B at 4°C for 1 h (or 30 min at room temperature) and the membranes were then washed twice with TBS-T and once with TBS and developed with chemiluminesence reagents according to the manufacturer's protocol (Amersham).

Dot-blot far-Western blotting

Recombinant ASF/SF2 was treated or not with SRPK1 under kinase conditions, and aliquots were added directly to nitrocellulose filters. Filters subsequently were treated as for the far-Western filters, except that 3 μg of rp32 was used in the presence of 60 μg of GOD. For His-tagged ASF/SF2 detection, filters were blocked as above and monoclonal anti-His antibody (Clonetech) was added at 1:200 dilution in TBS-T-B for 30 min at room temperature. All steps proceeded as above, except that sheep anti-mouse was used at 1:2000.

Western blotting

For p32 detection, 30 μg of nuclear extract or 50 μg of cytoplasmic extract and 10 pmol of recombinant p32 were subject to 12% SDS–PAGE under reducing conditions. Blotting was done under standard conditions (Burnette, 1981). Membranes were blocked in TBS-T-DM,

washed three times in TBS and a 1:500 dilution of rabbit polyclonal anti-p32 (Matthews and Russell, 1998) was added in TBS-T + 1% BSA for 12–16 h at 4°C. They were then washed three times in TBS-T and donkey anti-rabbit Ig (Amersham) was incubated at 1:2000 dilution in TBS-T at 4°C for 1 h, followed by washing twice with TBS-T, once in TBS, and were developed with chemiluminesence reagents according to the manufacturer's protocol (Amersham).

In vitro kinase assay

The indicated amounts of recombinant ASF/SF2 and ΔRS proteins were incubated at room temperature with no or increasing amounts of recombinant p32 for 5 min, followed by a further 15 min incubation at room temperature with SRPK1 (Gui *et al*., 1994), HeLa-NE (0.5 μg) or Clk/Sty (Colwill *et al*., 1996). The kinase buffer used contained: 20 mM Tris pH 7.4, 130 mM KCl, 15 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM ATP and 1 μCi of [γ-^{32}P]ATP. Products were resolved on 12 or 15% reducing SDS–PAGE and subjected to autoradiography.

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