The adenovirus E4-ORF4 splicing enhancer protein interacts with a subset of phosphorylated SR proteins

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SR proteins purified from uninfected HeLa cells inhibit adenovirus IIIa pre-mRNA splicing by binding to the intronic IIIa repressor element (3RE). In contrast, SR proteins purified from late adenovirus-infected cells are functionally inactivated as splicing repressor proteins by a virus-induced dephosphorylation. We have shown that the adenovirus E4-ORF4 protein, which binds the cellular protein phosphatase 2A (PP2A) and activates IIIa splicing *in vitro* and *in vivo*, induces SR protein dephosphorylation. Here we show that E4-ORF4 interacts with only a subset of SR proteins present in HeLa cells. Thus, E4-ORF4 interacts efficiently with SF2/ASF and SRp30c, but not with other SR proteins. Interestingly, E4-ORF4 interacts with SF2/ASF through the latter's RNA recognition motifs. Furthermore, E4-ORF4 interacts preferentially with the hyperphosphorylated form of SR proteins found in uninfected HeLa cells. E4-ORF4 mutant proteins that fail to bind strongly to PP2A or SF2/ASF do not relieve the repressive effect of HeLa SR proteins on IIIa pre-mRNA splicing in transient transfection experiments, suggesting that an interaction between all three proteins is required for E4-ORF4-induced SR protein dephosphorylation.

Keywords: adenovirus/E4-ORF protein/protein phosphatase 2A/splicing enhancer/SR proteins

Introduction

The adenovirus E4-ORF4 protein is a 14 kDa protein that binds to the B55 and B56 subunits of protein phosphatase 2A (PP2A) (Kleinberger and Shenk, 1993; Shtrichman *et al.*, 2000). PP2A is a serine/threonine phosphatase consisting of a core dimer of the A subunit and the catalytic C subunit, which interacts with a family of regulatory B subunits (Kamibayashi *et al.*, 1994; McCright and Virshup, 1995; Csontos *et al.*, 1996). A

wealth of data implicate PP2A in the regulation of a number of cellular functions, but the mechanisms by which B subunits target the core dimer to different substrates are not well understood (reviewed in Wera and Hemmings, 1995; Millward *et al.*, 1999). The E4-ORF4–PP2A complex reduces E1A activation of AP-1 transcription factor activity (Muller *et al.*, 1992). This reduction in transcription factor activity correlates with an increased accumulation of underphosphorylated forms of E1A and c-Fos. E4-ORF4 also inhibits E1A activation of junB (Kleinberger and Shenk, 1993), and adenovirus E2 (Mannervik *et al.*, 1999) and E4 transcription (Bondesson *et al.*, 1996). E4-ORF4 has also been shown to induce p53-independent apoptosis (Lavoie *et al.*, 1998; Marcellus *et al.*, 1998; Shtrichman and Kleinberger, 1998) by a mechanism requiring E4-ORF4 binding to PP2A (Marcellus *et al.*, 2000; Shtrichman *et al.*, 2000). We have shown further that E4-ORF4 regulates adenovirus L1 splicing, and induces SR protein dephosphorylation (Kanopka *et al.*, 1998). Collectively, available evidence suggests that E4-ORF4 regulates transcription, splicing and apoptosis through its binding to PP2A. Potentially the effect of E4-ORF4 on apoptosis is the result of its regulatory role on transcription factor and/or splicing factor activity.

The adenovirus L1 unit is an example of a gene whose last intron is spliced using a common 5' splice site and two competing 3' splice sites, generating two predominant cytoplasmic L1 mRNAs: the 52,55K (proximal 3' splice site) or the IIIa (distal 3' splice site) mRNAs, respectively (reviewed in Imperiale *et al.*, 1995). L1 splicing is regulated during the infectious cycle. Thus, the 52,55K mRNA is the only L1 mRNA produced early after infection, whereas the IIIa mRNA becomes the predominant L1 mRNA accumulating late after infection (Chow *et al.*, 1979; Akusjärvi and Persson, 1981; Nevins and Wilson, 1981; Svensson and Akusjärvi, 1986). We have shown previously that SR proteins purified from HeLa cells (SR-HeLa) inhibit IIIa splicing by binding to a purine-rich intronic repressor element, the 3RE, located immediately upstream of the IIIa branch site (Kanopka *et al.*, 1996). SR protein binding to the 3RE blocks splicing by inhibiting U2 snRNP recruitment to the IIIa branch site.

SR proteins are a family of essential splicing factors required for constitutive splicing and functioning as regulators of alternative splicing (reviewed in Fu, 1995; Manley and Tacke, 1996; Graveley, 2000). SR proteins have one or two N-terminal RNA recognition motifs (RRMs) and a C-terminus rich in arginine/serine dipeptide repeats (the RS domain), which mediates protein interactions with other components of the splicing machinery (Zahler *et al.*, 1992; Cáceres and Krainer, 1993; Wu and

Maniatis, 1993; Zuo and Manley, 1993; Kohtz *et al.*, 1994).

SR proteins are highly phosphorylated *in vivo* (Roth *et al.*, 1990), mainly in the RS domain (Colwill *et al.*, 1996), a modification that is required for their function in spliceosome assembly (Mermoud *et al.*, 1994; Roscigno and Garcia-Blanco, 1995; Xiao and Manley, 1997). Thus, phosphorylated SR proteins are needed for initiation of spliceosome assembly, and SR protein dephosphorylation appears to be required for splicing catalysis (Cao *et al.*, 1997). On the other hand, the level of SR protein phosphorylation appears to be critical, since both hyperphosphorylation and hypophosphorylation inhibit their activity as activators of splicing (Kanopka *et al.*, 1998; Prasad *et al.*, 1999), in both constitutive and activated alternative splicing. The phosphorylated status of SR proteins has also been shown to control their localization and transport in the nucleus between speckles and sites of active transcription and splicing. Thus, phosphorylation appears to release SR proteins from their storage sites and target them to sites of transcription and splicing in the nucleus (Colwill *et al.*, 1996; Caceres *et al.*, 1998; Misteli *et al.*, 1998).

We have shown previously that, during an adenovirus infection, SR proteins become functionally inactivated as IIIa splicing repressor proteins by a virus-induced dephosphorylation (Kanopka *et al.*, 1998). This post-translational modification reduces their capacity to bind to the 3RE and, as a consequence, alleviates their repressive effect on IIIa splicing. We further showed that the virus-encoded E4-ORF4 protein induces SR protein dephosphorylation, and has the capacity to activate IIIa pre-mRNA splicing, both *in vitro* and in transiently transfected cells (Kanopka *et al.*, 1998).

Here we determine the binding specificity of E4-ORF4 interaction with SR proteins. Much to our surprise, we find that E4-ORF4 interacts specifically with only a subset of the SR proteins expressed in HeLa cells. Thus, E4-ORF4 interacts efficiently with SF2/ASF (also named ASF/SF2) and SRp30c, but not with SRp20, SC35, SRp40, SRp55 or SRp75. The specificity appears to result from the fact that E4-ORF4 associates with SF2/ASF through the latter's RRM1. Furthermore, E4-ORF4 selectively interacts with the hyperphosphorylated form of SR proteins. Previously we showed that the 3RE is necessary for E4-ORF4 activation of IIIa splicing (Kanopka *et al.*, 1998). Here we extend this finding by demonstrating that the 3RE is sufficient for E4-ORF4-mediated activation of splicing. Moreover, we show that E4-ORF4 mutant proteins that show reduced binding to SF2/ASF or inefficient recruitment of an active PP2A phosphatase complex do not activate IIIa splicing in transient transfection experiments.

Results

E4-ORF4 interacts selectively with SF2/ASF and SRp30c

To study the specificity of E4-ORF4 interaction with SR proteins, we incubated GST-E4-ORF4 with ³⁵S-labeled SR proteins produced by coupled *in vitro* transcription-translation. As shown in Figure 1, glutathione S-transferase (GST)-E4-ORF4 interacts efficiently with SF2/ASF and SRp30c, but only weakly, if at all, with

Fig. 1. E4-ORF4 interacts selectively with SF2/ASF and SRp30c. GST or GST-E4-ORF4 was incubated with the indicated ³⁵S-labeled SR proteins. Bound proteins were separated by gel electrophoresis and visualized by autoradiography.

SRp20, SC35, SRp40, SRp55 or SRp75 in a GST pull-down assay. Interestingly, the RS domain of SF2/ASF is not important for this interaction, since SF2-ΔRS interacts at least as efficiently as wild-type SF2/ASF with E4-ORF4 (Figure 2B, lanes 1–6). In contrast, deletion of RRM1 of SF2/ASF abolished binding (Figure 2B, lanes 7–9).

The requirement for the RRM1 of SF2/ASF for E4-ORF4 interaction was confirmed further *in vivo*. For this experiment, 293 cells were co-transfected with plasmids encoding wild-type E4-ORF4 and T7-tagged SF2/ASF mutant proteins. As shown in Figure 2C, immunoprecipitation with an anti-E4-ORF4 antibody co-immunoprecipitated wild-type SF2/ASF and SF2-ΔRS, but not SF2-RRM1/RS or SF2-RRM2/RS, confirming the *in vitro* binding data (Figure 2B). Furthermore, E4-ORF4 did not interact with SC35 *in vivo* (Figure 2C, lanes 13 and 14), reinforcing the conclusion from the *in vitro* binding studies (Figure 1) suggesting that E4-ORF4 interaction with SF2/ASF is specific. Probing the western blot with anti-E4-ORF4 demonstrated that E4-ORF4 was expressed similarly in all transfections (data not shown).

The observation that the RRM1 of SF2/ASF are required for interaction with E4-ORF4 could indicate that E4-ORF4 becomes tethered indirectly to SF2/ASF through simultaneous, but independent, interaction with contaminating RNA in the extract. However, RNase treatment of the *in vitro* translated ³⁵S-labeled SF2/ASF did not disrupt the interaction with E4-ORF4 *in vitro* (Figure 2B, lanes 13–15) or *in vivo* (Figure 2C, lanes 11–12), suggesting that the SF2/ASF interaction with E4-ORF4 is direct. Also, the observation that SRp20, SC35, SRp40, SRp55 and SRp75, which would also be expected to bind RNA in the TNT extract, did not interact with E4-ORF4, further supports our conclusion that RNA does not unspecifically mediate the E4-ORF4 interaction with SF2/ASF.

Collectively, our results show that E4-ORF4 interacts efficiently with a subset of HeLa SR proteins: SF2/ASF and SRp30c. Moreover, E4-ORF4 appears to associate specifically with SF2/ASF through its RRM1.

E4-ORF4 interacts with hyperphosphorylated SR proteins

SR proteins are highly phosphorylated *in vivo*, a modification that is important for their function in spliceosome assembly and splicing catalysis (reviewed in Misteli, 1999). Since E4-ORF4 induces SR protein dephosphorylation, E4-ORF4 might be expected to interact preferentially with hyperphosphorylated SR proteins. To

Fig. 2. E4-ORF4 interacts with SF2/ASF through its RNA recognition motifs. **(A)** Schematic illustration showing the structure of SF2/ASF mutant proteins used in **(B)** and **(C)**. **(B)** An *in vitro* binding assay using GST-E4-ORF4 and 35 S-labeled SF2/ASF wild-type and mutant proteins. In lanes 13–15, the *in vitro* transcribed/translated 35 S-labeled SF2/ASF extract was treated with 5 μ g of RNase A for 15 min at 37°C before incubation with GST-E4-ORF4. **(C)** Extracts prepared from 293 cells co-transfected with pCMV-E4-ORF4 and plasmids encoding T7-tagged wild-type or mutant SF2/ASF proteins or SC35 were immunoprecipitated with an anti-E4-ORF4 antibody. Proteins were separated by gel electrophoresis and transferred to a membrane. SF2/ASF and SC35 proteins were detected using an anti-T7 antibody in a western blot assay. In lanes 11 and 12, the cell extract was treated with RNase A before immunoprecipitation with E4-ORF4.

test this hypothesis, we compared the capacity of E4-ORF4 to interact with SR-HeLa and SR proteins purified from late adenovirus-infected cells (SR-Ad). For this experiment, equal amounts of SR-HeLa and SR-Ad were separated by gel electrophoresis and transferred to a membrane. As shown in Figure 3A, probing the filter with the phospho-epitope-specific monoclonal antibody 104 (mAb104; Zahler *et al.*, 1992) demonstrated that SR-HeLa proteins were recognized efficiently by mAb104, whereas SR-Ad were not (lanes 1 and 4). Interestingly, incubating SR-Ad with a small amount of HeLa cell nuclear extract (HeLa-NE) restored the phospho-epitope(s) recognized by mAb104 to SR-Ad (lane 5), whereas incubation in HeLa-NE pre-treated with hexokinase and glucose to deplete the small ATP pool present in a dialyzed extract did not restore the phospho-epitope(s) (lane 6). The small amount of HeLa-NE used in this phosphorylation assay did not contribute detectable amounts of SR proteins (data not shown). Note that the proteins recognized by mAb104 in SR-Ad migrate slightly faster than SR-HeLa (compare the SRp30 fraction in lanes 1 and 4), a result that is compatible with the conclusion that the SR proteins recognizable by mAb104 in SR-Ad are hypophosphorylated. Also, incubating SR-Ad in HeLa-NE resulted in a reduction in mobility such that SR-Ad migration was identical to that of SR-HeLa (lane 5). Taken together, these results support our previous conclusion that SR-Ad proteins are hypophosphorylated (Kanopka *et al.*, 1998), and furthermore suggest that SR-Ad can be reconverted to SR proteins with the same electrophoretic mobility and mAb104-recogniz-

able phospho-epitope(s) as SR-HeLa by *in vitro* phosphorylation.

To measure total SF2/ASF levels, we reprobed the filter with mAb96 (Hanamura *et al.*, 1998), which recognizes RRM1 of SF2/ASF. As shown in Figure 3B, two distinct populations of SF2/ASF, with clear differences in migration, can be detected in SR-HeLa and SR-Ad, respectively (compare lanes 1 and 4). The slower migrating species is recognized by mAb104. Treatment of SR-HeLa with calf intestinal alkaline phosphatase resulted in an accumulation of the faster migrating species observed in SR-Ad (data not shown). We conclude that the two species represent hyperphosphorylated and unphosphorylated forms of SF2/ASF. Interestingly, the weak mAb104 signal seen in the SRp30 fraction in SR-Ad (Figure 3A, lane 4) migrates at a position between the highly and unphosphorylated SF2/ASF, a result strengthening the conclusion that SR-Ad is hypophosphorylated (Kanopka *et al.*, 1998). The results extend our previous results by suggesting that most of SF2/ASF is unphosphorylated in late adenovirus-infected cells. Furthermore, the results show that incubation of SR-Ad in HeLa-NE produces a quantitative shift in mobility of SF2/ASF to the slower migrating species seen in SR-HeLa (compare lanes 4 and 5).

Interestingly, reprobing the filter with GST-E4-ORF4 in a far western blot assay showed that E4-ORF4 interacts efficiently only with the SRp30 fraction in SR-HeLa but not in SR-Ad (Figure 3C, lanes 1 and 4). Importantly, *in vitro* phosphorylation of SR-Ad restored E4-ORF4 interaction to the slower, mAb104-recognizable species

Fig. 3. E4-ORF4 interacts preferentially with hyperphosphorylated SR proteins. (A) SR proteins prepared from HeLa cells (SR-HeLa, lane 1) and adenovirus-infected HeLa cells (SR-Ad, lane 4) were pre-incubated in nuclear extract (HeLa-NE) or nuclear extract depleted of ATP [HeLa-NE(-ATP)] (lanes 2, 3, 5 and 6). Samples were separated on a 10% SDS-polyacrylamide gel, transferred to a membrane and probed with mAb104 (an SR protein-specific antibody that detects phospho-epitopes in the RS domain) in a western blot assay. (B) The filter was probed with mAb96 (an SF2/ASF-specific antibody that detects the RRM1 of SF2/ASF) in a western blot assay. (C) The filter was probed with GST-E4-ORF4 in a far western blot assay. E4-ORF4-interacting proteins were visualized by chemiluminescence using an anti-E4-ORF4 antibody. Note that the autoradiograms shown are taken from different experiments.

(Figure 3C, lane 5). Collectively, these results suggest that E4-ORF4 interacts preferentially with hyperphosphorylated SR proteins, and support our binding studies (Figure 1) by indicating that E4-ORF4 interacts selectively with the SRp30 fraction, which contains SF2/ASF and probably SRp30c. It is noteworthy that E4-ORF4 also interacts with a cluster of slower migrating proteins, in a phosphorylation-dependent manner (Figure 3C). However, these interactions are only seen in reactions containing HeLa-NE (Figure 3C, lanes 2, 3, 5 and 6), suggesting that they represent E4-ORF4 interactions with other host cell proteins. Coomassie Blue staining of identical gels showed that equal amounts of SR proteins were analyzed in each sample (data not shown).

The 3RE is necessary and sufficient for E4-ORF4 activation of splicing

We have shown previously that the 3RE is necessary for E4-ORF4 activation of IIIa pre-mRNA splicing in a transient transfection assay (Kanopka *et al.*, 1998). Thus, replacing the 49 nucleotide 3RE with the corresponding sequence from the rabbit β -globin first intron abolished E4-ORF4 stimulation of IIIa splicing. More recently we have shown that IIIa splicing is also regulated by a second viral element, the IIIa virus infection-dependent splicing enhancer (the 3VDE; Mühlemann *et al.*, 2000). This finding prompted us

Fig. 4. The 3RE is sufficient to confer an E4-ORF4-enhanced splicing phenotype to β -globin. (A) Schematic representation of the IIIa- β -globin hybrid reporter transcripts used. (B) Quantitative S1 analysis of IIIa- β -globin hybrid mRNA levels in HeLa cells co-transfected with pCMV-E4-ORF4 and the reporter plasmids depicted in (A). Average splicing efficiencies and standard deviations are shown (three experiments).

to re-examine whether the 3RE was the only element required for E4-ORF4 activation of splicing. For this experiment, we reconstructed eukaryotic expression vectors for some of the IIIa- β -globin hybrid constructs previously used in our *in vitro* studies (Kanopka *et al.*, 1996). As shown in Figure 4, transfer of the 3RE to β -globin significantly reduced β -globin splicing [transcript glob (3RE), compare lanes 1 and 3]. This finding is in agreement with our *in vitro* experiments showing that the 3RE is the minimal sequence element required for SR protein inhibition of splicing (Kanopka *et al.*, 1996). Importantly, co-transfection of a plasmid encoding E4-ORF4 stimulated β -globin splicing and annulled the repressive effect of the 3RE (compare lanes 1 and 4). In agreement with our previous demonstration that the 3VDE only functions efficiently in the context of virus-infected cells (Mühlemann *et al.*, 2000), transfer of the 3VDE to β -globin resulted in a severe inhibition of β -globin splicing [transcript glob (3VDE), lanes 5]. However, compared with glob (3RE) splicing, glob (3VDE) splicing was only marginally activated by E4-ORF4 co-transfection (lane 6). Collectively, our data show that the 3RE is the primary element conferring an E4-ORF4-enhanced splicing phenotype, and show furthermore that the 3RE is both necessary (Kanopka *et al.*, 1998) and sufficient for E4-ORF4 activation of splicing (Figure 4).

	E4-ORF4	R1	R2	A11	R3-9	12	p32	R4	R3-2	A3	A10
SF2/ASF binding*	+	-	+	+	+	-	(+)	(+)	(+)	-	+
PP2A recruitment*	+	-	-	+	+	+	(+)	+	-	-	(+)
Activation of IIIa pre-mRNA splicing	+	-	-	+	-	-	-	-	-	-	-

Fig. 6. Strong E4-ORF4 interaction with SF2/ASF, and E4-ORF4 recruitment of a functional phosphatase complex correlate with E4-ORF4 activation of IIIa pre-mRNA splicing. The figure presents a summary of E4-ORF4 mutant protein interactions with SF2/ASF (Figure 5B), E4-ORF4 recruitment of a functional phosphatase complex (Shtrichman *et al.*, 1999) and the capacity of E4-ORF4 mutant proteins to activate IIIa pre-mRNA splicing in a transient transfection assay (Figure 5C). *The percentage binding or recruitment are as follows: -, 0–30%; (+), 30–60%; and +, 60–100%.

The E4-ORF4 mutant analysis shows an almost perfect correlation between strong E4-ORF4 interaction with SF2/ASF, E4-ORF4 recruitment of an active phosphatase complex and IIIa splicing activation (Figure 6). Thus, strong interaction between E4-ORF4 and only one component is not enough to activate IIIa splicing in a transient transfection assay. For example, mutant 12 recruits an active phosphatase complex but does not interact efficiently with SF2/ASF, and is defective in IIIa pre-mRNA splicing activation. Mutant R2 shows the opposite binding specificity, i.e. it binds SF2/ASF but does not recruit an active phosphatase, and it is similarly defective in activation of splicing. Moreover, mutants with reduced binding affinity for SF2/ASF or reduced phosphatase recruitment do not stimulate IIIa pre-mRNA splicing, whereas mutant A11, which interacts efficiently with both SF2/ASF and PP2A, is a strong activator of IIIa pre-mRNA splicing. The only E4-ORF4 mutant protein that does not conform to this general rule is mutant R3-9, which does not activate IIIa splicing despite the fact that it interacts efficiently with SF2/ASF *in vitro* and recruits an active phosphatase complex (Figure 6). We do not know the significance of this observation. However, we note that E4-ORF4 binding to PP2A and E4-ORF4 recruitment of an active phosphatase complex similarly did not show a perfect match (Shtrichman *et al.*, 1999). Thus, E4-ORF4 binding to PP2A is not synonymous with active dephosphorylation. The simplest explanation for this discrepancy between binding and dephosphorylation is that the E4-ORF4–PP2A interaction requires a higher order structure that correctly presents the target protein to the PP2A catalytic subunit. Thus, mutant R3-9 interaction with SF2/ASF and PP2A may be non-productive and fail to present the SR protein correctly for catalysis. However, we cannot exclude the possibility that E4-ORF4 targets another important splicing factor that is required for E4-ORF4 function in splicing.

The good correlation between E4-ORF4–PP2A–SF2/ASF interaction and IIIa pre-mRNA splicing activation (Figure 6) suggests that SF2/ASF is a major SR protein targeted by E4-ORF4. Although E4-ORF4 also interacts with SRp30c, this interaction does not show such a strict correlation with IIIa splicing activation. Thus, all tested E4-ORF4 mutant proteins (Figure 5A) bind with essentially the same efficiency to SRp30c (data not shown). Taken together, these results indicate that SRp30c, which is probably a minor species compared with the abundant

SF2/ASF (Hanamura *et al.*, 1998), does not make a significant contribution to E4-ORF4-induced activation of IIIa pre-mRNA splicing, at least not in HeLa cells.

The observation that E4-ORF4 interacts specifically with SF2/ASF through its RRM (Figure 2) provides an explanation for the observed selective interaction of E4-ORF4 with only a subset of SR proteins (Figure 1). However, the observation that E4-ORF4 interacts preferentially with hyperphosphorylated SR proteins (Figure 3) may seem paradoxical. Why does E4-ORF4 interact efficiently with SF2- Δ RS, which lacks the RS domain containing the serines that are phosphorylated? The answer to this problem appears to lie in the fact that SF2/ASF is also phosphorylated at residues located in the RRM in HeLa-NE (Petersen-Mahrt *et al.*, 1999). Also, Clk/Sty phosphorylate SF2/ASF outside the RS domain (Colwill *et al.*, 1996), further corroborating the idea that SF2/ASF is not only phosphorylated in the RS domain. Thus, E4-ORF4 may induce RS domain dephosphorylation, but binding to SF2/ASF may be mediated through phosphorylated residues in the SF2/ASF RRM. Alternatively, RS domain dephosphorylation may perturb the structure of SF2/ASF such that E4-ORF4 cannot bind.

We have shown previously that the cellular protein p32, similarly to E4-ORF4, regulates IIIa pre-mRNA splicing, and inhibits SF2/ASF as a splicing factor (Petersen-Mahrt *et al.*, 1999). Interestingly, both E4-ORF4 and p32 appear to interact with SF2/ASF through residues located in the RRM and, 'at a distance', affect RS domain phosphorylation. Thus, E4-ORF4 and p32 both induce accumulation of hypophosphorylated SR proteins. However, they do so by opposite mechanisms. E4-ORF4 activates dephosphorylation while p32 accomplishes the same by inhibiting phosphorylation (reviewed in Akusjärvi, 1999).

Collectively, our results suggest that two proteins regulating SF2/ASF function interact with SF2/ASF through its RRM and regulate RS domain function. Hypothetically, this observation may provide an explanation for the finding that SF2/ASF chimeric proteins, created by fusing RS domains from other SR proteins to the SF2/ASF RRM, functionally substitute for SF2/ASF *in vivo* (Wang *et al.*, 1998). Thus, if regulatory proteins controlling SF2/ASF phosphorylation specifically interact with SF2/ASF through its RRM, the origin of the RS domain may be secondary for SF2/ASF regulation.

The observation that E4-ORF4 interacts selectively with only some HeLa SR proteins was surprising, and is potentially very significant. Thus, we showed previously that all SR proteins, except SRp20, are underphosphorylated in late adenovirus-infected cells (Kanopka *et al.*, 1998). The finding that E4-ORF4 does not interact with all SR proteins, therefore, suggests that there must be other viral proteins that contribute to the accumulation of hypophosphorylated SR proteins in late virus-infected cells. In this respect, it is interesting to note that we have shown recently that the viral E4-ORF6 protein, which functions as an alternative splicing factor *in vivo* (Nordqvist *et al.*, 1994), activates IIIa pre-mRNA splicing *in vitro*, in a reaction requiring the SR-responsive 3RE (S.Fan and G.Akusjärvi, in preparation). Potentially, the E4-ORF6 protein may be the missing viral factor controlling SR protein phosphorylation. However, the

mechanistic details of how E4-ORF6 activates IIIa splicing remain to be established.

Materials and methods

Plasmids and proteins

Detailed plasmid maps and sequences are available upon request, and some are found at <http://www.bmc.uu.se/IMIM/res/GA.html>. Plasmids for transfection (pCMV variants) and *in vitro* binding studies (pGST variants) of E4-ORF4 and derivatives encoding mutant E4-ORF4 proteins have been described (Kleinberger and Shenk, 1993; Shtrichman *et al.*, 1999). Reporter plasmids pAdCMV-glob, pAdCMV-glob (3RE) and pAdCMV-glob (3VDE) were generated by recloning the relevant fragments from previously described plasmids 1, 3 and 4 (Figure 1 in Kanopka *et al.*, 1996) into the *Bam*HI site in plasmid pAdCMV-BamHI vector (Molin *et al.*, 1998). Reporter plasmid pAdCMV-IIIa has been described (Kanopka *et al.*, 1998). Plasmids encoding SRp20, SRp30c, SRp40, SRp55 and SRp75 and those encoding T7-tagged SF2/ASF, SF2- Δ RS, SF2-RRM2/RS, SF2-RRM1/RS, SF2-RS and SC35 have been described (Caceres and Krainer, 1993; Screaton *et al.*, 1995; Caceres *et al.*, 1997). 35 S-labeled SR proteins were made by *in vitro* transcription-translation using the TNT system (Promega), from PCR templates amplified using a 5' primer, containing a T7 promoter and the first 15 bases of the respective SR protein gene, and a common 3' primer, complementary to β -globin sequences in the plasmid sequence downstream of the protein insert. GST and GST fusion proteins were expressed and purified from *Escherichia coli* as described by the manufacturer (Pharmacia Biotech). SR proteins were purified from HeLa cells (SR-HeLa) and late adenovirus-infected HeLa cells (SR-Ad) as described (Zahler *et al.*, 1992; Kanopka *et al.*, 1998).

In vitro binding assay

In vitro translated 35 S-labeled SR proteins were incubated with GST, GST-E4-ORF4 or GST-E4-ORF4 mutant proteins on glutathione-Sepharose beads for 2 h at 4°C in TBS buffer (20 mM Tris-HCl pH 7.5, 139 mM NaCl) containing 0.15% Tween-20, washed twice in the binding buffer, and separated on a 12% SDS-polyacrylamide gel under reducing conditions. As a standard, half of the 35 S-labeled SR protein extract used in the GST binding assay was run in parallel on the gel.

Cells and transient transfections

HeLa and 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% newborn calf serum and 100 U/ml penicillin-streptomycin (Life Technologies). Subconfluent monolayers of cells grown on 60 mm dishes were transfected using the FuGene cocktail (Boehringer-Mannheim) or the calcium phosphate co-precipitation technique (Ausubel *et al.*, 1987). Cells were harvested 46 h post-transfection and lysed in IsoB-NP40 (10 mM Tris-HCl pH 7.9, 0.15 M NaCl, 1.5 mM MgCl₂, 0.5% NP-40) on ice. Cytoplasmic RNA was prepared by phenol/chloroform/isoamyl-alcohol (25:24:1) extractions. To prepare soluble extracts for immunoprecipitation, cells were incubated for 30 min on ice in lysis buffer [50 mM Tris-HCl pH 7.4, 250 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.5% NP-40, protease inhibitor mix (Boehringer Mannheim)]. Cell debris was removed by centrifugation at 13 500 g for 10 min.

Immunoprecipitations and western blotting

A 3 μ g aliquot of pCMV-E4-ORF4 or empty vector (pCMV) and 1 μ g of plasmids encoding T7-tagged SF2/ASF, SF2- Δ RS, SF2-RRM1/RS, SF2-RRM2/RS or SC35 were co-transfected into 293 cells. Soluble cell lysates were incubated with anti-E4-ORF4 antibody covalently coupled to protein A-Sepharose (Pharmacia Biotech) at 4°C overnight. Immunoprecipitates were washed three times in lysis buffer and separated on a 12% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane by electroblotting and probed with anti-E4-ORF4 or anti-T7 (Novagen) antibodies in a standard western blot assay (Ausubel *et al.*, 1987). Antibody interaction with proteins on the membrane was visualized by chemiluminescence (Amersham).

Western and far western analysis of SR protein

SR-HeLa or SR-Ad proteins (0.5 μ g) were incubated for 10 min at room temperature in either the absence or presence of 5 μ g of HeLa cell nuclear extract (HeLa-NE) or HeLa-NE depleted of ATP [HeLa-NE(-ATP)], separated on a 10% SDS-polyacrylamide gel and electroblotted to a

nitrocellulose membrane. The filter was blocked in TBS buffer containing 0.05% Tween-20, 0.5 mM β -glycerophosphate and 5% milk for 30 min at room temperature. The filter was then incubated with 2 μ g/ml GST-E4-ORF4 in TBS buffer containing 0.05% Tween-20 and 1% bovine serum albumin (BSA) for 30 min at room temperature. The filter was washed in TBS containing 0.05% Tween-20, and probed in the first round with an anti-E4-ORF4 antibody (Shtrichman *et al.*, 1999), which was visualized by chemiluminescence as described by the manufacturer (Amersham). The filter was stripped by incubation in 0.78% mercaptoethanol, 62.5 mM Tris-HCl, 2% SDS for 20 min at 50°C and reprobed in the second round with mAb104 (Zahler *et al.*, 1992), and in a third round with mAb96 (Hanamura *et al.*, 1998).

ATP depletion

HeLa-NE (5 μ g) was mixed with 2 mM glucose, 2.5 mM MgCl₂ and 0.7 U of hexokinase (Boehringer Mannheim) in a total volume of 20 μ l, and incubated for 10 min at 30°C. The amount of hexokinase and glucose required for complete depletion of the ATP pool in dialyzed nuclear extracts was determined in control experiments containing 1 μ Ci of [γ - 32 P]ATP.

S1 nuclease assays

A 3 μ g aliquot of reporter plasmids pAdCMV-IIIa, pAdCMV-glob, pAdCMV-glob (3RE) or pAdCMV-glob (3VDE) and 0.2 μ g of pCMV plasmids encoding wild-type or mutant E4-ORF4 proteins were co-transfected into HeLa cells. Total cytoplasmic RNA was isolated 46 h post-transfection and 10 μ g subjected to S1 analysis (Ausubel *et al.*, 1987) using the IIIa-specific 5'-end-labeled DNA probe previously described (Kanopka *et al.*, 1998), or a β -globin-specific 5'-end-labeled DNA probe. After S1 nuclease digestion, the products were precipitated and separated on an 8% denaturing polyacrylamide gel and the protected fragments visualized and quantitated by PhosphorImager scanning.

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